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(74) Agent: **WILSON, Mary, J.**; Nixon & Vanderhye P.C.,
1100 North Glebe Road, Suite 800, Arlington, VA 22201-
4714 (US).

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(71) Applicant (for all designated States except US): **DUKE UNIVERSITY** [US/US]; Box 90083, Durham, NC 27708-0083 (US).

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(54) Title: IMMUNOGEN

(57) Abstract: The present invention relates, in general, to an immunogen and, in particular, to an immunogen for inducing antibodies that neutralize a wide spectrum of HIV primary isolates. The invention also relates to a method of inducing anti-HIV antibodies using same.

IMMUNOGEN

This a continuation-in-part of Application No. 10/664,029, filed September 17, 2003, which is a continuation-in-part of Application No. 09/960,717, 5 filed September 24, 2001, which claims priority from Provisional Application No. 60/234,327, filed September 22, 2000, Provisional Application No. 60/285,173, filed April 23, 2001, Provisional Application No. 60/323,697, filed September 21, 2001 10 and from Provisional Application No. 60/323,702, filed September 21, 2001, the entire contents of which are incorporated herein by reference.

TECHNICAL FIELD

The present invention relates, in general, to an 15 immunogen and, in particular, to an immunogen for inducing antibodies that neutralize a wide spectrum of HIV primary isolates. The invention also relates to a method of inducing anti-HIV antibodies using such an immunogen.

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BACKGROUND

As the HIV epidemic continues to spread worldwide, the need for an effective HIV vaccine remains urgent. A key obstacle to HIV vaccine development is the extraordinary variability of HIV and the rapidity 25 and extent of HIV mutation (Wain-Hobson in The

Evolutionary biology of Retroviruses, SSB Morse Ed.
Raven Press, NY, pgs 185-209 (1994)).

Myers, Korber and colleagues have analyzed HIV sequences worldwide and divided HIV isolates into 5 groups or clades, and provided a basis for evaluating the evolutionary relationship of individual HIV isolates to each other (Myers et al (Eds), Human Retroviruses and AIDS (1995), Published by Theoretical Biology and Biophysics Group, T-10, Mail Stop K710, 10 Los Alamos National Laboratory, Los Alamos, NM 87545). The degree of variation in HIV protein regions that contain CTL and T helper epitopes has also recently been analyzed by Korber et al, and sequence variation documented in many CTL and T helper epitopes among HIV 15 isolates (Korber et al (Eds), HIV Molecular Immunology Database (1995), Published by Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM 87545).

A new level of HIV variation complexity was 20 recently reported by Hahn et al by demonstrating the frequent recombination of HIV among clades (Robinson et al, J. Mol. Evol. 40:245-259 (1995)). These authors suggest that as many as 10% of HIV isolates are mosaics of recombination, suggesting that vaccines 25 based on only one HIV clade will not protect immunized subjects from mosaic HIV isolates (Robinson et al, J. Mol. Evol. 40:245-259 (1995)).

The present invention relates to an immunogen suitable for use in an HIV vaccine. The immunogen will induce broadly cross-reactive neutralizing antibodies in humans and neutralize a wide spectrum of 5 HIV primary isolates.

SUMMARY OF THE INVENTION

The present invention relates to an immunogen for inducing antibodies that neutralize a wide spectrum of 10 HIV primary isolates. The invention also relates to a method of inducing anti-HIV antibodies using such an immunogen.

Objects and advantages of the present invention will be clear from the description that follows.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows CCR5 or CXCR4 co-receptor proteins (or their protein or peptide fragments) and soluble CD4 bound to gp160.

Figure 2 shows vesicles or liposomes containing 20 CD4, CCR5 (or CXCR4) bound to gp160 in a vesicle, liposome or an inactivated virion.

Figure 3 shows soluble CD4 and peptides reflective of CCR5 or CXCR4 gp120 binding sites bound to gp160.

5 Figure 4 shows B1Acore measurements of binding interactions between soluble gp120 of HIV isolate DH12 and soluble CD4.

Figure 5 shows binding interactions between HIV 89.6, JRFL, and DH12 soluble gp120 proteins and soluble CD4.

10 Figure 6 shows kinetic and binding affinities of the interactions between gp120 and CD4.

Figure 7 shows binding of soluble CD4 to gp120-expressing membranes from the CHO-wt (HIV-IIIB gp160-expressing) cells.

15 Figure 8 shows changes in gp160 and gp140 associated with a structure of gp160 that corresponds with the ability to induce cell fusion.

20 Figure 9 shows "freezing" of fusogenic epitopes upon addition of DP-178 or T649 peptides that are parts of the coiled coil region and that, when added

to CD4-triggered envelope, result in prevention of fusion.

Figures 10A-10D show binding of CD4 and CCR5 N-terminal peptide to HIV 89.6 gp140 oligomers.

5 Fig. 10A. Overlay of the binding curves of interactions between immobilized CD4 and HIV envelope proteins (89.6 gp120 and gp140). Data show that sCD4 bound to cleaved HIV 89.6 gp140 (cl) and to HIV 89.6 gp120 proteins. The negative control (negative ctrl) 10 was generated by injecting 89.6 gp120 proteins over a CD8 immobilized surface. Compared to HIV JRFL and DH12 gp120 env proteins, HIV 89.6 gp120 env bound to CD4 with a relatively higher affinity ($K_d = 146\text{nM}$, 96nM and 23nM for JRFL, DH12 and 89.6 env respectively). These 15 differences in K_d were predominantly due to differences in the off-rate ($k_d = 8.8 \times 10^{-4} \text{ s}^{-1}$, $4.7 \times 10^{-4} \text{ s}^{-1}$, $1.1 \times 10^{-4} \text{ s}^{-1}$ for JRFL, DH12 and 89.6 gp120 envelope proteins, respectively).

Fig. 10B. Binding of uncleaved CM235 gp140 (uncl) and IIIB-gp160 (uncl) oligomers to immobilized 20 CD4. Data show that whereas uncleaved IIIB-gp160 (Fig. 10B) oligomers bound poorly to CD4, uncleaved HIV CM235 gp140 (Fig. 10B) oligomers did bind sCD4. The negative control is the same injection of IIIB- 25 gp160 flowing over a surface immobilized with IIIB-gp160 proteins.

Fig. 10C. CD4-gp140 complexes (indicated by arrow) were first formed by injecting soluble 89.6 gp140 oligomers over a CD4-immobilized surface. Then the CCR5 N-terminal peptide (D1) was injected to 5 monitor its binding to CD4-gp140 complexes.

Fig. 10D. An overlay of the binding curves of CCR5 N-terminal peptide, D1, binding to 89.6 gp140 oligomers in the presence and absence of CD4. On a CD4-gp140 complex, the D1 peptide binds with an 10 apparent K_d of 280nM ($k_a=9\times 10^3 \text{ M}^{-1}\text{s}^{-1}$, $k_d= 2.56\times 10^{-3} \text{ s}^{-1}$). In the absence of CD4, the D1 peptide binds constitutively to cleaved gp140 oligomers with a similar on-rate but a faster off-rate ($k_d=0.01 \text{ s}^{-1}$, $K_d= 1\mu\text{M}$). The difference in the steady-state binding 15 of CCR5-D1 peptide in the presence and absence of CD4 is a quantitative effect. To ensure that all gp140 molecules were bound to CD4, cleaved gp140 proteins were captured on a CD4 immobilized sensor surface (RU=530), and soluble 89.6 gp140 proteins were 20 directly immobilized (RU=3300) on an adjacent flow cell of the same sensor chip.

Figures 11A-11D show CD4 and CCR5 extracellular domain peptides induce binding of the HR-2 peptides to HIV 89.6 gp140.

25 Fig. 11A. Binding of HR-2 peptide, DP178 following binding of 89.6 gp140 oligomers to CD4

(solid line), CD4 and CCR5-D1 peptide (broken line), CCR5-D1 peptide (solid circle) and to gp140 oligomers alone (broken lines).

Fig. 11B. Binding of the HR-2 peptide, T649QL to 5 89.6 gp140 oligomers (broken line), CD4-gp140 complex (open circle) and to CD4-gp140-D1 complex (solid line). 89.6 gp140 proteins and CCR5-D1 peptide were sequentially injected over a sCD4 immobilized surface (see Fig. 10C).

10 Fig. 11C. Binding of HR-2 peptides, DP178 and a control scrambled DP178 peptides to CD4-89.6 gp140 complexes.

Fig. 11D. Kinetics data for the binding of HR-2 peptides, DP178 and T649QL to 89.6 gp140 after 15 induction with CD4 and the CCR5-D1 peptide. Rm refers to the RU bound at steady-state during the injection of the same conc of HR-2 peptides (5mM) over the indicated gp140 complexes. nm= rate constants could not be measured due to extremely low affinity binding 20 of HR-2 peptides to gp140 proteins.

Figure 12 shows immunoprecipitation by HR-2 peptide and western blot analysis of 89.6 gp140 envelope proteins. Biotinylated HR-2 peptide, DP178 constitutively immunoprecipitated both cleaved gp41 25 and uncleaved gp140 proteins. The level of immunoprecipitated gp140 and gp41 was augmented by sCD4. HIV 89.6 gp140 proteins were incubated with 2.5

μg of biotinylated DP178 in the presence (Lanes 1 and 5) or absence (Lanes 3 and 7) of sCD4 (2 μg). As controls, HIV 89.6 gp140 proteins were also incubated with 2.5 μg of biotinylated scrambled DP178 peptide in 5 the presence (Lanes 2 and 6) or absence (Lanes 4 and 8) of sCD4 (2 μg). Lane 9 shows gp140 and gp41 within the gp140 preparation immunoblotted with mab 7B2 (anti-gp41), while Lane 10 shows gp120 within the gp140 preparation reactive with mab T8 (anti-gp120).

10 Figure 13 shows the induced binding of HR-2 peptides to soluble HIV 89.6 gp140 envelope.

Figures 14A-14F show induction of HR-2 peptide binding to 89.6 gp140 by soluble CD4. Fig. 14A. Capture of soluble 89.6 gp140 over a CD4 (solid lines) 15 and T8 (broken lines) immobilized surface. Roughly 3000 to 5000 RU of sCD4 and T8 mab were immobilized on a CM5 sensor chip. Soluble 89.6 gp140 proteins were then captured to the same level on both surfaces and are labeled CD4-gp140 and T8-gp140 respectively. Figs. 14B and 14C. Binding of the 19b (Fig. 14B) and 20 2F5 (Fig. 14C) antibodies to CD4-gp140 (solid line) and T8-gp140 (broken line) complexes. After stabilization of gp140 capture, either the 19b (anti-gp120 V3) or 2F5 (anti-gp41) antibodies (300μg/ml) 25 were injected to assess the relative amounts of gp120 and gp41 on both surfaces. Figs. 14D-14F. Binding of

HR-2 peptide, DP178 (Figs. 14D), scrambled DP178 (Figs. 14E) and T649Q26L (Figs. 14E) to CD4-gp140 (solid line) or T8-gp140 (broken line) surfaces. 50 μ g/ml of each HR-2 peptide was injected at 30 μ l/min 5 over both CD4 and T8 surfaces.

Figures 15A-15E show binding interactions of HR-2 peptides with CD4-gp140 and CD4-gp120 complexes.

Figs. 15A and 15B. Overlay of the binding curves showing the interactions between HR-2 peptides, DP178 (Fig. 15A) and T649Q26L (Fig. 15B), and CD4-gp120 (broken lines) and CD4-gp140 (solid lines). Figs. 15A and 15B show the titration of the HR-2 peptide DP178 and T649Q26L between 12.5 to 100 μ g/ml over CD4-gp140 (solid lines) and CD4-gp120 (broken lines) surfaces.

Fig. 15C. There was no binding of scrambled DP178 HR-2 peptide to either CD4-gp140 or T8-gp140. Fig. 15D. CD4 induced HR-2 peptide binding to gp120 envelope proteins. There was no specific binding of HR-2 peptides to the T8-gp120 complex, while the sCD4-gp120 and sCD4-gp140 surfaces did stably bind HR-2 peptides. 20 Compared to CD4-gp120, higher binding was observed with CD4-gp140. Table in Fig. 15E shows the binding rate constants and dissociation constants for the HR-2 peptides and CD4-gp120 and CD4-gp140 complexes. Data 25 are representative of 2 separate experiments. HR-2 peptides were injected at 30 μ l/min simultaneously over a blank, sCD4 and a sCD4-gp120 or sCD4-gp140

surfaces. The curves presented show specific binding to CD4-gp120 or CD4-gp140 and were derived after subtraction of binding signals from blank and sCD4 surfaces.

5 Figures 16A-16F show constitutive binding of HR-2 peptides to immobilized gp41 and CD4 induced binding to gp140. Fig. 16A and 16B. Binding of DP178 (12.5 to 100 μ g/ml) to immobilized ADA gp41 and CD4-gp140 (Fig. 16B) surfaces. Figs. 16C and 16D. Binding of 10 scrambled DP178 (12.5 to 100 μ g/ml, Fig. 16D) to immobilized ADA gp41 and the binding of HR-2 peptide, DP178 (12.5 to 50 μ g/ml) to T8-gp140 surfaces. Figs. 16E and 16F. Scatchard plots of RU vs RU/C for DP178 binding to gp41 (Fig. 16E) and to CD4-gp140 (Fig. 16F). Data are representative of 2 separate 15 experiments.

Figures 17A and 17B show induction of HR-2 peptide binding to gp120-gp41 complex. Binding of HR-2 peptide DP178 (50 μ g/ml) to immobilized gp41, gp41-20 gp120 and gp41-gp120-CD4 complexes. Compared to gp41 surface, higher binding of DP178 is observed on gp41-gp120 and gp41-gp120-CD4 surfaces. Soluble 89.6 gp120 (100 μ g/ml) or a gp120-CD4 mixture (gp120 pre-25 incubated with 0.3 mg/ml of CD4 for 30 min) was injected at 5 μ l/min for 10 min over a sensor surface immobilized with ADA gp41. At the end of the injections, wash buffer (PBS) was allowed to flow

until the surface was stable with no baseline drift. Roughly 300 RU of gp120 and 850 RU of gp120-CD4 formed stable complexes with the immobilized gp41 surface. The gp120-CD4 binding was subsequently reduced to 5 about 325 RU (to be roughly equivalent to gp41-gp120 surface) with short injections of a regeneration buffer (10mM glycine-HCl, pH 2.0), followed by an injection of gp120-CD4 mixture. Thus both surfaces were stable and had equivalent amount of associated 10 gp120 or gp120-CD4.

Figure 18. Soluble CD4 binding to HIV gp120 non-covalently linked to gp41 results in binding of HR-2 to gp120 and upregulation of gp41-gp120 association. An immunogen is cross-linked CD4-gp120-gp41 in soluble 15 form.

Figure 19. A32 binding to HIV gp120 non-covalently linked to gp41 results in binding of HR-2 to gp120 and upregulation of gp41-gp120 association. An immunogen is cross-linked A32 (whole Ig) -gp120-gp41 20 in soluble form or A32 (Fab or Fab2) -gp120-gp41 in soluble form.

Figure 20. Soluble CD4 binding to soluble gp120 results in exposure of the CCR5 binding site, and the gp41 HR-2 binding site.

Figure 21. A32 mab binding to soluble HIV gp120 results in exposure of the CD4 binding site and the CCR5 binding site.

Figure 22. Soluble CD4 binding to soluble
5 uncleaved HIV gp140 upregulates HR-2 binding to HR-1 in gp41, and exposes the CCR5 binding site. An immunogen is crosslinked CD4-gp140 with or without DP178 or T649Q26L bound.

Figure 23. Mab A32 binding to soluble uncleaved
10 HIV gp140 upregulates HR-2 binding to HR-1 in gp41, and exposes the CCR5 binding site. An immunogen is crosslinked A32 or a fragment thereof-gp140 with or without DP178 or T649Q26L bound to gp41 or elsewhere in the complex.

15 Figure 24. Soluble CD4 binding to HIV gp120 non-covalently linked to gp41 results in binding of HR-2 to gp120 and upregulation of gp41-gp120 association. An immunogen is cross-linked CD4-gp120-gp41 in soluble form, with HR-2 peptides bound to HR-
20 1.

Figure 25. A32 binding to HIV gp120 non-covalently linked to gp41 results in binding of HR-2 to gp120 and upregulation of gp41-gp120 association. An immunogen is cross-linked A32 (whole Ig)-gp120-gp41
25 in soluble form or A32 (Fab or Fab2)-gp120-gp41 in

soluble form, with HR-2 peptide bound to HR-1 or elsewhere in the complex.

Figure 26. Induction of HR-2 peptide binding to HIV 89.6 gp140 by soluble CD4. Soluble 89.6 gp140 proteins were captured to the same level on all three surfaces and are labeled CD4-gp140, T8-gp140 and A32-gp140. Figure shows binding of HR-2 peptide DP178 to T8-gp140 (circle), CD4-gp140 (solid line) and A32-gp140 (broken line) complexes. 50 μ g/ml of HR-2 peptide was injected at 30 μ l/min over A32, CD4 and T8 surfaces. Only specific binding, after bulk responses and non-specific binding of HR-2 peptides to sCD4, T8 and A32 surfaces were subtracted, are shown.

Figures 27A-27I show A32 mAb and sCD4 induced HR-2 peptide binding to soluble HIV-1 Env gp120 proteins using the "capture assay". A schematic of the 'capture assay' is shown in Fig. 27I. Rectangular bars represent the surface of the sensor chip on which sCD4 or mAbs A32 or T8 were covalently immobilized. Soluble gp120 proteins were first captured on each of these surfaces and then DP178 or sDP178 peptides were injected and their binding interactions were monitored. (Fig. 27A) Capture of soluble 89.6 Env gp120 proteins on an A32 mAb immobilized surface. Approximately 2000 RU (Response Unit) of 89.6 gp120 was bound to immobilized A32 and after washing, DP178

peptide (50 μ g/ml) was injected at 30 μ l/min. Based on their molecular size (1:27), 2000 RU of immobilized gp120 would bind 74 RU of DP178 peptide. Dotted line represents the background binding obtained by 5 injecting DP178 over the A32 surface prior to capture of gp120 proteins. Arrows point to start of injection of gp120 and DP178. (Figs. 27B-27C) In Fig. 27B, specific and non-specific signals were obtained by injecting DP178 (50 μ g/ml) over A32-gp120 (solid) and 10 A32 (broken line) surfaces. In Fig. 27C, specific binding signal (solid) is shown after subtraction of non-specific signal (broken line). Similarly, non-specific binding to both T8 and sCD4 immobilized surfaces was also subtracted. (Figs. 27D-27E) Binding 15 of DP178 to 89.6 gp120 captured on T8 (solid triangles), CD4 (open triangles) and A32 (solid line). 50 μ g/ml of DP178 peptide was flowed at 30 μ l/min over each of these surfaces after 2000 RU of 89.6 gp120 was bound (Fig. 27D). In Fig. 27E, these same surfaces 20 were also used to monitor binding of scrDP178 (50 μ g/ml). Marked induction of DP178 binding to 89.6 gp120 was observed on CD4 and A32 surfaces, but not on a T8 surface. (Fig. 27F) Binding of DP178 and DP107 peptides to A32-gp120 complex. 50 μ g/ml of DP178 or 25 DP107 peptide was flowed at 30 μ l/min over 89.6 gp120 captured on a A32 surface. Overlay of curves shows specific binding of gp120 to DP178, but not to the HR-1 control peptide, DP107. Data are a representative

experiment of 3 performed. (Figs. 27G-27H) Binding of HR-2 peptide DP178 to BaL gp120 captured on T8 (solid triangles), CD4 (open triangles) and A32 (solid line). 5 50 μ g/ml of DP178 peptide was flowed at 30 μ l/min over each of these surfaces after RU of BaL gp120 was bound (Fig. 27G). These same surfaces were also used to monitor binding of scrDP178 (50 μ g/ml) (Fig. 27H).

Figures 28A-28E show A32 induced binding of gp120 10 to immobilized DP178 using the "streptavidin (SA) - chip assay". A schematic of the assay is shown in Fig. 28E. Biotinylated DP178 (Fig. 28A), scrDP178 (Fig. 28B) and scrDP107 (not shown) were attached to a SA-sensor chip through biotin-streptavidin 15 interactions. 200-300 RU of each peptide was attached to individual flow cells of the SA chip. The scrDP107 surface was used as in-line reference subtraction of non-specific and bulk effect. All data shown here were derived after subtraction of signals from this 20 reference surface. In Fig. 28A, 89.6 gp120 (-A32, line with closed circle), mAb A32 (dotted line) and gp120 pre-incubated with A32 mAb (+A32, solid line) were injected over all three surfaces at 30 μ l/min. Compared to sDP178 surface (Fig. 28B), gp120 pre- 25 incubated with mAb A32 showed enhanced binding to DP178 surface (Fig. 28A). An overlay of gp120+A32 curves from DP178 (dotted line) and scrDP178 (closed circle) is shown in Fig. 28C. The solid line

represents resultant binding curves from DP178 surface obtained after subtraction of binding signal from scrDP178 surface (closed circle). The baseline curve (with closed triangle) was derived from a similar 5 subtraction of scrDP178 binding. The Table on the right shown dissociation rates (off-rates) obtained by curve fitting to a single component dissociation (AB= A + B) and parallel dissociation of two independent complexes (A₁B= A₁+B; A₂B= A₂+B) models to the -A32 10 and +A32 curves respectively from Fig. 28A. Data are a representative experiment of 3 performed.

Figures 29A-29C show precipitation gp120 Env proteins by HR-2 peptide and analysis by Western blots. (Fig. 29A) HIV-1 89.6 gp120 proteins (40 μ g) 15 were incubated with 12.5 μ g of biotinylated DP178 (lanes 1,3) or scrDP178 (lanes 2,4) in the presence (lanes 1, 2) or absence (lanes 3, 4) of mAb A32 (18 μ g), followed by precipitation with streptavidin- 20 agarose beads. In the presence of mAb A32, lane 1 shows markedly higher amounts of precipitated gp120 proteins compared to the corresponding lane 2, in the absence of A32. (Fig. 29B) The blot shown in Fig. 29B was prepared under the conditions described for blot 25 Fig. 29A except that lanes 1 and 2 were blotted with mAb T8 after preincubation of gp120 with sCD4 (14 μ g) and followed by precipitation with biotinylated DP178 or scrDP178 as above. As observed for A32 mAb, the

presence of sCD4 caused marked increase in the amount of gp120 precipitated. (Fig. 29C) The means +/- SEM of density of blotted bands in lanes 1 (in presence of A32 or sCD4) and 3 (in the absence of A32 or sCD4) are plotted for blots Fig. 29A and Fig. 29B. Equivalent areas of each band in lanes 1 and 3 (for DP178) and lanes 2 and 4 (for scrDP178) were scanned and volumes determined in OD units/mm². The non-specific OD unit values from scrDP178 (lanes 2 and 4) were then subtracted from those of DP178 (lanes 1 and 3). The mean density of precipitation with DP178 in the presence of A32 (solid bar, +A32) was significantly higher (* p< 0.005) than those in its absence (hatched bar, -A32). Similarly, the mean band density of precipitated gp120 in the presence of sCD4 (solid bar, +CD4) was significantly higher (**p=0.001) than gp120 precipitated in the absence of CD4 (hatched bars, -CD4). Data was derived from representative experiment of 3 (with sCD4) and 6 (with A32) performed.

Figures 30A-30G show effect of anti-gp120 mAbs on DP178 binding to gp120. (Fig. 30A) mAb A32 (solid line, +A32) induced the binding of HIV-1 89.6 gp120 to DP178, while the neutralizing mAb 2G12 (closed circle, +2G12) had no effect on the binding signal and showed binding level comparable to constitutive binding of 89.6 gp120 to DP178 (closed triangle, -2G12). The broken line shows the signal obtained with mAb 2G12

flowing over DP178. (Fig. 30B) 17b mAb failed to induce the binding of 89.6 gp120 to DP178 (triangle, +17b), and in mAb 17b presence the binding signal was slightly lower than those obtained in mAb 17b absence (solid line, -17b) or the background signal from mAb 17b alone (broken line). (Fig. 30C) Addition of mAb 17b to A32-gp120 complexes completely blocked the binding of 89.6 gp120 to DP178. Compared to 89.62 gp120 binding to DP178 (close circles, -A32), mAb A32 induced a 7-fold increase in binding of gp120 to DP178 (solid line, +A32). When 17b mAb was added to 89.6 gp120 pre-incubated with A32, DP178 binding to gp120 was completely blocked (broken line, +A32+17b). Thus, mAb 17b can block A32 induced binding of DP178 to 89.6 gp120. (Fig. 30D) mAb 17b Fab inhibits the binding of 89.6 gp120 bound to A32 surface. Increasing concentrations (0.0 to 25 μ g/ml) of mAb 17b Fab was pre-incubated with a fixed concentration of 89.6 gp120 (100 μ g/ml) and then this gp120-17b complex was bound to an A32 immobilized surface. DP178 at 50 μ g/ml was injected at 30 μ l/min. (Fig. 30E) Both mAb 48d (anti-CCR5 binding site mAb) and 19b (anti-V3 mAb) have inhibitory effects on the binding of DP178 to gp120 bound to A32 mAb. 89.6 gp120 (100 μ g/ml) was pre-incubated with either 2G12, 19b or 48d mAbs at 1:1 molar ratio and then each of these solutions were bound to the A32 immobilized surface. DP178 at 50 μ g/ml was injected at 30 μ l/min. (Fig. 30F)

Relative blocking effects of mAbs on DP178 binding. The bar plot and the mean RU binding was derived from 3 independent sets of data similar to those shown in Fig. 30E. %RU of DP178 binding was calculated by 5 taking the RU binding of DP178 in the absence of any blocking antibody as 100%. Data shown in Figs. 30A-30C were derived using the 'SA-chip assay', while the 'capture assay' was used for data shown in Fig. 30D-30F. Data are representative of 3 separate experiments 10 performed. (Fig. 30G) Schematic.

Fig. 31A-31F. Blocking of DP178 binding to 89.6 gp120 by C4 peptides. Overlay of binding of DP178 to 89.6 gp120 bound to mAb A32 in the absence (solid line) or presence of either C4-V3_{89.6P} (Fig. 31A), C4-15 V3_{MN} (Fig. 31B), C4-scrV3 (Fig. 31C), C4 (Fig. 31D) or V3 (Fig. 31E) peptide. 89.6 gp120 was bound to mAb A32 and binding of DP178 was monitored as described in Fig. 27. HR-2 peptide DP178 was pre-incubated with each of these peptides and then injected over A32- 20 gp120 surface. Specific binding of DP178 (50 mg/ml) to A32-gp120 in the presence of blocking peptides (50mg/ml) is shown with solid circles. Data are representative of 3 separate experiments performed. Data presented for C4-V3_{89.6P} and C4-V3_{MN} are taken 25 from two separate experiments. (Fig. 31F) Schematic.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an immunogen that induces broadly reactive neutralizing antibodies that are necessary for an effective AIDS vaccine. In 5 one embodiment, the immunogen comprises a cleaved or uncleaved gp140 or gp160 HIV envelope protein that has been "activated" to expose intermediate conformations of conserved neutralization epitopes that normally are only transiently or less well exposed on the surface 10 of the HIV virion. The immunogen is a "frozen" triggered form of HIV envelope that makes available specific epitopes for presentation to B lymphocytes. The result of this epitope presentation is the production of antibodies that broadly neutralize HIV.

15 The concept of a fusion intermediate immunogen is consistent with observations that the gp41 HR-2 region peptide, DP178, can capture an uncoiled conformation of gp41 (Furata et al, *Nature Struct. Biol.* 5:276 (1998)), and that formalin-fixed HIV-infected cells 20 can generate broadly neutralizing antibodies (LaCasse et al, *Science* 283:357 (1997)). Recently a monoclonal antibody against the coiled-coil region bound to a conformational determinant of gp41 in HR1 and HR2 regions of the coiled-coil gp41 structure, but did not 25 neutralize HIV (Jiang et al, *J. Virol.* 10213 (1998)). However, this latter study proved that the coiled-coil

region is available for antibody to bind if the correct antibody is generated.

Conserved neutralization sites on the HIV envelope can be on two regions; they can be on gp41 5 and they can be on gp120.

The regions and conformations of gp41 that are exposed during gp140 or gp160 "triggering" ("activation") can be expected to be conserved since: i) the amino acid sequences of the coiled-coil region 10 are conserved and ii) the function of the fusogenic envelope complex is conserved and essential for virus pathogenicity. This conservation is key to the production of broadly neutralizing anti-HIV antibodies.

15 The immunogen of one aspect of the invention comprises HIV envelope cleaved or uncleaved gp140 or gp160 either in soluble form or anchored, for example, in cell vesicles made from gp140 or gp160 expressing cells, or in liposomes containing translipid bilayer 20 HIV gp140 or gp160 envelope. Alternatively, triggered gp160 in aldrithio 1-2 inactivated HIV-1 virions can be used as an immunogen. The gp160 can also exist as a recombinant protein either as gp160 or gp140 (gp140 is gp160 with the transmembrane region and possibly 25 other gp41 regions deleted). Bound to gp160 or gp140 can be recombinant CCR5 or CXCR4 co-receptor proteins (or their extracellular domain peptide or protein fragments) or antibodies or other ligands that bind to

the CXCR4 or CCR5 binding site on gp120, and/or soluble CD4, or antibodies or other ligands that mimic the binding actions of CD4 (Figure 1). Alternatively, vesicles or liposomes containing CD4, CCR5 (or CXCR4) (Figure 2), or soluble CD4 and peptides reflective of CCR5 or CXCR4 gp120 binding sites (Figure 3).

Alternatively, an optimal CCR5 peptide ligand can be a peptide from the N-terminus of CCR5 wherein specific tyrosines are sulfated (Bormier et al, Proc. Natl.

10 Acad. Sci. USA 97:5762 (2001)). The data in Figure 13 clearly indicate that the triggered immunogen may not need to be bound to a membrane but may exist and be triggered in solution. Alternatively, soluble CD4 (sCD4) can be replaced by an envelope (gp140 or gp160) 15 triggered by CD4 peptide mimotopes (Vitra et al, Proc. Natl. Acad. Sci. USA 96:1301 (1999)). Other HIV co-receptor molecules that "trigger" the gp160 or gp140 to undergo changes associated with a structure of gp160 that induces cell fusion can also be used. (See 20 also Fig. 8.) The data presented in Example 2 demonstrate that ligation of soluble HIV gp140 primary isolate HIV 89.6 envelope with soluble CD4 (sCD4) induced conformational changes in gp41 (see Fig. 13).

In one embodiment, the invention relates to an 25 immunogen that has the characteristics of a receptor (CD4)-ligated envelope with CCR5 binding region exposed but unlike CD4-ligated proteins that have the CD4 binding site blocked, this immunogen has the CD4

binding site exposed (open). Moreover, this immunogen can be devoid of host CD4, which avoids the production of potentially harmful anti-CD4 antibodies upon administration to a host. (See Figures 18-25.)

5 The immunogen can comprise gp120 envelope ligated with a ligand that binds to a site on gp120 recognized by an A32 monoclonal antibodies (mab) (Wyatt et al, J. Virol. 69:5723 (1995), Boots et al, AIDS Res. Hum. Retro. 13:1549 (1997), Moore et al, J. Virol. 68:8350 (1994), Sullivan et al, J. Virol. 72:4694 (1998), Fouts et al, J. Virol. 71:2779 (1997), Ye et al, J. Virol. 74:11955 (2000)). One A32 mab has been shown to mimic CD4 and when bound to gp120, upregulates (exposes) the CCR5 binding site (Wyatt et al, J. Virol. 69:5723 (1995)). Ligation of gp120 with such a ligand also upregulates the CD4 binding site and does not block CD4 binding to gp120. Advantageously, such ligands also upregulate the HR-2 binding site of gp41 bound to cleaved gp120, uncleaved gp140 and cleaved gp41, thereby further exposing HR-2 binding sites on these proteins - each of which are potential targets for anti-HIV neutralizing antibodies.

20 In a specific aspect of this embodiment, the immunogen comprises soluble HIV gp120 envelope ligated with either an intact A32 mab, a Fab2 fragment of an A32 mab, or a Fab fragment of an A32 mab, with the result that the CD4 binding site, the CCR5 binding site and the HR-2 binding site on gp120 are

exposed/upregulated. The immunogen can comprise gp120 with an A32 mab (or fragment thereof) bound or can comprise gp120 with an A32 mab (or fragment thereof) bound and cross-linked with a cross-linker such as .3% 5 formaldehyde or a heterobifunctional cross-linker such as DTSSP (Pierce Chemical Company). The immunogen can also comprise uncleaved gp140 or a mixture of uncleaved gp140, cleaved gp41 and cleaved gp120. An A32 mab (or fragment thereof) bound to gp140 and/or 10 gp120 or to gp120 non-covalently bound to gp41, results in upregulation (exposure) of HR-2 binding sites in gp41, gp120 and uncleaved gp140. Binding of an A32 mab (or fragment thereof) to gp120 or gp140 also results in upregulation of the CD4 binding site 15 and the CCR5 binding site. As with gp120 containing complexes, complexes comprising uncleaved gp140 and an A32 mab (or fragment thereof) can be used as an immunogen uncross-linked or cross-linked with cross-linker such as .3% formaldehyde or DTSSP. In one 20 embodiment, the invention relates to an immunogen comprising soluble uncleaved gp140 bound and cross linked to a Fab fragment of an A32 mab, optionally bound and cross-linked to an HR-2 binding protein.

The gp120 or gp140 HIV envelope protein triggered 25 with a ligand that binds to the A32 mab binding site on gp120 can be administered in combination with at least a second immunogen comprising a second envelope, triggered by a ligand that binds to a site distinct

from the A32 mab binding site, such as the CCR5 binding site recognized by mab 17b. The 17b mab (Kwong et al, *Nature* 393:648 (1998) available from the AIDS Reference Repository, NIAID, NIH) augments sCD4 binding to gp120. This second immunogen (which can also be used alone or in combination with triggered immunogens other than that described above) can, for example, comprise soluble HIV gp120 envelope ligated with either the whole 17b mab, a Fab2 fragment of the 17b mab, or a Fab fragment of the 17b mab. It will be appreciated that other CCR5 ligands, including other antibodies (or fragments thereof), that result in the CD4 binding site being exposed can be used in lieu of the 17b mab. This further immunogen can comprise gp120 with the 17b mab, or fragment thereof, (or other CCR5 ligand as indicated above) bound or can comprise gp120 with the 17b mab, or fragment thereof, (or other CCR5 ligand as indicated above) bound and cross-linked with an agent such as .3% formaldehyde or a heterobifunctional cross-linker, such as DTSSP (Pierce Chemical Company). Alternatively, this further immunogen can comprise uncleaved gp140 present alone or in a mixture of cleaved gp41 and cleaved gp120. Mab 17b, or fragment thereof (or other CCR5 ligand as indicated above) bound to gp140 and/or gp120 in such a mixture results in exposure of the CD4 binding region. The 17b mab, or fragment thereof, (or other CCR5 ligand as indicated above)-gp140 complexes can be

present uncross-linked or cross-linked with an agent such as .3% formaldehyde or DTSSP.

Soluble HR-2 peptides, such as T649Q26L and DP178 (see below), can be added to the above-described 5 complexes to stabilize epitopes on gp120 and gp41 as well as uncleaved gp140 molecules, and can be administered either cross-linked or uncross-linked with the complex.

A series of monoclonal antibodies (mabs) have 10 been made that neutralize many HIV primary isolates, including, in addition to the 17b mab described above, mab IgG1b12 that binds to the CD4 binding site on gp120 (Roben et al, J. Virol. 68:482 (1994), Mo et al, J. Virol. 71:6869 (1997)), mab 2G12 that binds to a 15 conformational determinant on gp120 (Trkola et al, J. Virol. 70:1100 (1996)), and mab 2F5 that binds to a membrane proximal region of gp41 (Muster et al, J. Virol. 68:4031 (1994)). A mixture of triggered envelope immunogens can be used to optimize induction 20 of antibodies that neutralize a broad spectrum of HIV primary isolates. Such immunogens, when administered to a primate, for example, either systemically or at a mucosal site, induce broadly reactive neutralizing antibodies to primary HIV isolates.

As indicated above, various approaches can be 25 used to "freeze" fusogenic epitopes in accordance with the invention. For example, "freezing" can be effected by addition of the DP-178 or T-649Q26L

peptides that represent portions of the coiled coil region, and that when added to CD4-triggered envelop, result in prevention of fusion (Rimsky et al, J. Virol. 72:986-993 (1998) (see Figures 9 and 13)). HR-2 peptide bound gp140, gp41 or gp160 can be used as an immunogen or crosslinked by a reagent such as DTSSP or DSP (Pierce Co.), formaldehyde or other crosslinking agent that has a similar effect (see below).

The data presented in Example 4 demonstrate that the fusion inhibitor peptide DP178 (T-20) binds to the CXCR4 binding site region of gp120 and that the binding is induced by sCD4 and by the anti-gp120 human monoclonal antibody A32. Accordingly, in a specific embodiment, the present invention relates to an immunogen comprising an HR-2 binding peptide (e.g., DP178) directly bound to gp120 at a CD4 inducible site. CD4 induction can be achieved with CD4 or a CD4 mimetic, such as a monoclonal antibody (e.g., A32) or other small molecule that binds the CD4 binding site.

"Freezing" can also be effected by the addition of 0.1% to 3% formaldehyde or paraformaldehyde, both protein cross-linking agents, to the complex, to stabilize the CD4, CCR5 or CXCR4, HR-2 peptide gp160 complex, or to stabilize the "triggered" gp41 molecule, or both (LaCasse et al, Science 283:357-362 (1999)).

Further, "freezing" of gp41 or gp120 fusion intermediates can be effected by addition of

heterobifunctional agents such as DSP (dithiobis[succimidylpropionate]) (Pierce Co. Rockford, ILL., No. 22585ZZ) or the water soluble DTSSP (Pierce Co.) that use two NHS esters that are 5 reactive with amino groups to cross link and stabilize the CD4, CCR5 or CXCR4, HR-2 peptide gp160 complex, or to stabilize the "triggered" gp41 molecule, or both.

Inherent differences exist in HIV isolates among HIV clades and among HIV isolates from patients in 10 varying geographic locations. Triggered complexes for HIV vaccine development can be made with HIV envelopes from a variety of HIV clades and from a variety of locations. Triggered complexes comprising antibodies or fragments thereof that upregulate the CCR5 binding 15 site, the CD4 binding site, or both, or antibodies, or fragments thereof, that are CD4 inducible can be produced by co-expressing in a dicistronic manner in a plasmid both gp120 and, for example, the heavy and light chain of the Fab region of the antibody, in 20 order to produce a recombinant protein that has the properties of the above described complexes.

The immunogen of the invention can be formulated with a pharmaceutically acceptable carrier and/or adjuvant (such as alum) using techniques well known in 25 the art. Suitable routes of administration of the present immunogen include systemic (e.g. intramuscular or subcutaneous). Alternative routes can be used when

an immune response is sought in a mucosal immune system (e.g., intranasal).

Certain aspects of the invention are described in greater detail in the non-limiting Example that 5 follows.

EXAMPLE 1

The feasibility of the immunogen production approach of the present invention has been shown using BiaCore 3000 technology. Figure 4 shows that CD4, not 10 CD8, can be bound to gp120. Figures 5, 6 show the differences in interaction of gp120 with the envelop of primary isolates and lab-adapted HIV strains. Figure 7 shows that vesicles from chimeric hamster ovary cells transfected with HIV-IIIB gp160 and that 15 these vesicles, present on a BiaCore LI Chip, show stabilized binding of soluble CD4 but not CD8.

EXAMPLE 2

Experimental Details

Proteins

20 Soluble, monomeric gp120 JRFL, gp120 DH12 and sCD4 were produced by Progenics, and were provided by the Division of AIDS, NIAID, NIH. HIVIIIB gp160 was obtained from Protein Sciences. Envelope proteins from HIV 89.6 (Clade B), and HIV CM235 (Clade E) 25 primary isolates were produced by Pat Earl, NIH, using

recombinant vaccinia viruses and purified as described (Earl et al, J. Virol. 68:3015-3026 (1994), Earl et al, J. Virol. 75:645-653 (2001)).

Briefly, BS-C-1 cells in 160 cm² flasks were
5 infected with vBD1(HIV 89.6 gp140) or vBD2 (gp120) vaccinia viruses. After 2h, the cells were washed in PBS and placed in serum-free OPTI-MEM media (Gibco) for 24-26 hr. The culture medium was then harvested by centrifugation and filtration (0.2μm) and then TX-100
10 was added to 0.5% (final conc., v/v). For some of the experiments, the culture medium was concentrated 15-30 fold and served as a source of multimeric gp140 glycoproteins (a mix of cleaved and uncleaved form). Further purification of these glycoproteins was
15 performed using a two-step procedure. In the first step, contaminating proteins were removed and glycoproteins from the medium were bound to a lentil lectin column and eluted with methyl α-D-mannopyranoside. This preparation contained ~50:50
20 cleaved and uncleaved gp140 and the per-purified culture supernatant concentrate are termed "cleaved gp140". Finally, oligomeric and dimeric gp140 were separated and purified by size exclusion chromatography. This gp140 preparation is termed
25 "uncleaved gp140". The glycoprotein fractions were pooled and concentrated using micro-concentrators.

Monoclonal Antibodies

Human monoclonal antibody against the gp120 V3 loop (19b) the CCR5 binding site (17b), and mab 7B2 against the immunodominant region of gp41 were the 5 gifts of James Robinson (Tulane University, New Orleans, LA). Mabs 2F5, IgG1b12 and 2G12 were obtained from the AIDS References Repository, NIAID, NIH.

CCR5 and HR-2 Peptides.

10 Synthetic peptides were synthesized (SynPep Corporation, Dublin, CA), and purified by reverse phase HPLC. Peptides used in this study had greater than 95% purity as determined by HPLC, and confirmed to be correct by mass spectrometry. The CCR5-D1 15 (MDYQVSSPIYDINYTSEPCQKINVQIAAR), peptide was derived from the N-terminus of human CCR5 (Bieniasz et al, EMBO Journal 16:2599-2609 (1997)). Gp41 peptides DP- 178 YTSLIHSILIEESQNQQEKNEQELLELDKWASLWNWF (Wild et al, Proc. Natl. Acad. Sci. USA 91:12676-12680 (1994)), T- 20 649 WMEWDREINNYTSЛИHSILIEESQNQQEKNEQELLEL (Rimsky et al, J. Virol. 72:986-993 (1998)), and T649-Q26L (WMEWDREINNYTSЛИHSILIEESQNQQEKNEQELLEL) (Shu et al, Biochemistry 39:1634-1642 (2000)) were derived from HIV-1 envelope gp41 from HIV 89.6 (Collmann et al, J. 25 Virol. 66:7517-7521 (1992)). As a control for HR-2 peptide binding, a scrambled sequence DP178 peptide was made as well.

Surface Plasmon Resonance Biosensor Measurements

SPR biosensor measurements were determined on a BIAcore 3000 (BIAcore Inc., Uppsala, Sweden) instrument. HIV envelope proteins (gp120, gp140, gp160) and sCD4 were diluted to 100-300 mg/ml in 10mM Na-Acetate buffer, pH 4.5 and directly immobilized to a CM5 sensor chip using standard amine coupling protocol for protein immobilization (Alam et al, Nature 381:616-620 (1996)). Binding of proteins and peptides was monitored in real-time at 25°C and with a continuous flow of PBS, pH 7.4 at 5-20ml/min. Analyte (proteins and peptides) were removed and the sensor surfaces were regenerated following each cycle of binding by single or duplicate 5-10 ml pulses of regeneration solution (10 mM glycine-HCl, pH 2.5 or 10mM NaOH).

All analyses were performed using the non-linear fit method of O'Shannessy et al. (O'Shannessy et al, Anal. Biochem. 205:132-136 (1992)) and the BIAevaluation 3.0 software (BIAcore Inc). Rate and equilibrium constants were derived from curve fitting to the Langmuir equation for a simple bimolecular interaction (A + B = AB).

In preliminary SPR experiments, it was determined that HIV gp120 envelope protein for HIV89.6 bound sCD4 most avidly with relatively little baseline drift ($t_{1/2}$ of binding, 105 min.) compared to HIV gp120 DH12 ($t_{1/2}$ of binding, 25 min) and HIV gp120 JRFL ($t_{1/2}$ of

binding, 14 min.). Thus, HIV89.6 gp120 and gp140 were produced for subsequent experiments.

5 Immunoprecipitation of HIV Envelope Proteins Followed by Western Blot Analysis. Soluble HIV 89.6 gp140 or gp120 proteins were incubated with or without 2 μ g of recombinant sCD4, and a dose range if either biotinylated DP178 or biotinylated scrambled DP178 as a control in a total volume of 50 μ l PBS for 2h

10 followed by incubation (4h) with 50 μ l suspension of streptavidin-agarose beads (Sigma Chemicals, St. Louis, MO). Immune complexes were washed x3 with 500 μ l of PBS, resuspended in SDS-PAGE sample buffer containing 2-ME, boiled for 5 min, and loaded onto

15 SDS-PAGE on 4-20% polyacrylamide gels. Gels were transferred to immunoblot membranes for Western blot analysis with either mabs T8 (anti-gp120 N-terminus) or 7B2 (anti-gp41 immunodominant region).

Results

20 *Binding of sCD4 to Cleaved and Uncleaved HIV Envelope.* Preliminary SPR studies showed that, of HIV gp120 envelopes JRFL, DH12 and 89.6, the HIV 89.6 gp120 demonstrated the most stable binding to sCD4 (see Figure 10A). Thus, preparations of HIV89.6 gp140

25 envelope proteins were studied that contained either cleaved or primarily uncleaved gp140 for binding to sCD4. Non-cleaved HIV CM235 gp140 and, as well,

cleaved HIV 89.6 gp140, were tested for their ability to bind sCD4 covalently anchored on the chip in the SPR biosensor assay. Cleaved 89.6 gp140 bound sCD4 well (Figure 10A). The uncleaved baculovirus-produced 5 HIV IIIB gp160 from Protein Sciences did not bind sCD4 (Mascola et al, J. Infect. Dis. 173:340-348 (1996)) (Figure 10B). However, cleavage of gp140 into gp120 and truncated gp41 was not an absolute requirement for binding of sCD4 to envelope preparations, as the 10 uncleaved Clade E envelope HIV CM235 gp140 also bound sCD4 (Figure 10B).

Binding of an N-terminal CCR5 Extracellular Domain Peptide to HIV 89.6 gp140 Envelope. The CCR5 binding site on HIV gp120 is inducible by sCD4 (Kwong et al, 15 Nature 393:648-659 (1998), Rizzuto et al, Science 280:1949-1953 (1998), Wyatt et al, Nature 393:705-710 (1998)). It was next determined if an N-terminal CCR5 extracellular domain synthetic peptide could be made 20 that bound to HIV 89.6 gp140. A 30 aa peptide was produced from the N-terminus of CCR5 (termed CCR5-D1), and tested for ability to bind to cleaved HIV 89.6 gp140.

Low levels of constitutive binding of the CCR5-D1 25 peptide to cleaved gp140 were found, while sCD4 binding to cleaved gp140 envelope induced more stable binding of CCR5-D1 binding to gp140 (Figures 10C and 10D). Doms et al have found that the affinity of

native CCR5 to sCD4 ligated gp120 in the context of membrane bound envelope was 500 μ M (Hoffman et al, Proc. Natl. Acad. Sci. 97:11215-11220 (2000)). The binding affinity of this CCR5-D1 N-terminal domain 5 peptide to sCD4-ligated gp140 was found to be ~280 μ M (Figure 10D).

Effect of Soluble CD4 and the CCR5-D1 Extracellular Domain Peptide on the Binding of HR-2 Peptides to Cleaved HIV 89.6 gp140 Envelope Proteins. A major goal of this study was to determine if fusion-associated conformations of gp41 could be detected using SPR assays of HR-2 peptide binding. It was reasoned that if HR-2 peptides can bind gp140, the 15 gp41 coiled-coil structure must be uncoiled, such that endogenous HR-2 is not bound to HR-1. Three such HR-2 peptides were used in this study, DP178 (Wild et al, Proc. Natl. Acad. Sci. USA 91:12676-12680 (1994), Rimsky et al, J. Virol. 72:986-993 (1998)), T-649 20 (Rimsky et al, J. Virol. 72:986-993 (1998)), and T649Q26L (Shu et al, Biochemistry 39:1634-1642 (2000)). DP178 contains C-terminal amino acids of HR-2 (Wild et al, Proc. Natl. Acad. Sci. USA 91:12676-12680 (1994), Rimsky et al, J. Virol. 72:986-993 25 (1998)) while T649Q26L contains more N-terminal amino acids of HR-2 (Rimsky et al, J. Virol. 72:986-993 (1998), Shu et al, Biochemistry 39:1634-1642 (2000)). Low level binding DP178 to cleaved HIV 89.6 gp140 was

found (Figure 11A), and DP178 control scrambled peptide did not bind at all (Figure 11C).

When binding of HR-2 peptides were determined on cleaved gp140 ligated with sCD4, DP178 HR-2 peptide binding was induced on cleaved gp140 by sCD4 (Figure 11A). Addition of the CCR5-D1 N-terminal peptide to sCD4-gp140 complexes did not significantly change the K_d of DP178 binding to gp140 (0.7 to $1.1\mu M$) but did slow the on rate of DP178 binding ($7.9 \times 10^{-3} M^{-1} s^{-1}$ to $3.47 \times 10^{-3} M^{-1} s^{-1}$) indicating possibly an induction of protein conformation change (Figures 11A, 11B and Table in Figure 11).

The T649Q26L HR-2 peptide was designed to be of higher affinity for binding to HR-1 by the Q26L substitution (Shu et al, Biochemistry 39:1634-1642 (2000)), and indeed, in this study, T649Q26L also bound to ligated, cleaved CD4-gp140 complexes. Soluble CD4 increased the maximal DP178 binding to cleaved gp140 at equilibrium (R_m) by ~10 fold (from an R_m of DP178 binding with no sCD4 of 35 RU (response units) to an R_m of 320 RU with sCD4) (Figure 11). The k_d , s^{-1} (off-rate) of binding for DP178 to cleaved gp140 after sCD4 was .0056. sCD4 binding to cleaved gp140 oligomers resulted in a $t_{1/2}$ of DP178 binding of 124 sec. The k_d of binding for DP178 to sCD4 and CCR5-D1 ligated cleaved gp140 89.6 was $1.1\mu M$. An additional control for the specificity of HR-2 peptide binding

was the demonstration that the DP178 HR-2 peptide did not bind to sCD4-ligated gp120.

Ability of Biotinylated HR-2 Peptide to

5 *Immunoprecipitate HIV Envelope Proteins.* To identify the components in gp140 to which HR-2 peptides bind in solution, gp140 envelope was immunoprecipitated with biotinylated DP178 HR-2 peptide, and then the proteins bound to biotinylated HR-2 were analyzed by Western blot analysis. Figure 12 shows the HIV envelope components present in cleaved HIV 89.6 gp140. Lane 10 shows gp120 and gp140, immunoblotted with anti-gp120 mab T8. Lane 9 shows uncleaved gp140 and gp41 immunoblotted with the anti-gp41 mab, 7B2. Figure 12 also shows that biotinylated HR-2 peptide, DP178, constitutively immunoprecipitated both cleaved gp41 (~33kd) and uncleaved gp140 in the absence of sCD4 (lane 3), and the level of gp140 and gp41 immunoprecipitated by biotinylated HR-2 was enhanced by sCD4 (lane 1). Lanes 5 and 7 show that the anti-gp120 mab T8 recognized the gp120 region of the gp140 protein immunoprecipitated by biotinylated HR-2. Control immunoprecipitations with scrambled biotinylated DP178 peptide did not immunoprecipitate significant levels of HIV envelope proteins (Lanes 2, 4, 6 and 8).

CD4-Induced Binding of HR-2 Peptides to HIV 89.6 gp140 Envelope Proteins. A major goal of this study was to determine if fusion associated conformations of gp41 could be detected using SPR assays of HR-2 peptide binding. It was reasoned that if HR-2 peptides can bind gp140, the gp41 coiled-coil structure must be uncoiled, such that endogenous HR-2 is not bound to HR-1. Two such HR-2 peptides were used in this study, DP178 (Wild et al, Proc. Natl. Acad. Sci. USA 10 91:12676-12680 (1994), Rimsky et al, J. Virol. 72:986-993 (1998)) and T649Q26L (Rimsky et al, J. Virol. 72:986-993 (1998), (Shu et al, Biochemistry 39:1634-1642 (2000)). DP178 contains C-terminal amino acids of HR-2 (Wild et al, Proc. Natl. Acad. Sci. USA 15 91:12676-12680 (1994), Rimsky et al, J. Virol. 72:986-993 (1998)) while T649Q26L contains more N-terminal amino acids of HR-2 (Rimsky et al, J. Virol. 72:986-993 (1998), (Shu et al, Biochemistry 39:1634-1642 (2000)).

20 HIV envelope gp120 proteins bind to sCD4 with a relatively high affinity (Myszka et al, Proc. Natl. Acad. Sci. USA 97:9026-9031 (2000), Collman et al, J. Virol. 66:7517-7521 (1992)). In preliminary studies, it was found that soluble HIV 89.6 gp120 protein bound 25 strongly to immobilized sCD4, with a K_d 23nM and with an off-rate of $1.1 \times 10^{-4} \text{ s}^{-1}$. Thus, a CD4 immobilized surface allowed a very stable capture of HIV envelope, and this approach has been used to assay HR-2 peptide

binding to sCD4 bound HIV 89.6 envelope proteins. To create equivalent surfaces of tethered gp140 and gp120 on CM5 sensor chips, sCD4 and anti-gp120 mab T8 immobilized on sensor chips were used as capture surfaces. A blank chip served as an in-line reference surface for subtraction of non-specific binding and bulk responses. Mab T8 bound HIV 89.6 gp120 with an affinity of 5.6 nM. Thus, both CD4 and the mab T8 provided stable surfaces for anchoring HIV envelope proteins.

Since HIV 89.6 gp140 contains both cleaved and uncleaved gp140, it was important to show that gp41 was present in CD4-gp140 complexes following capture of gp140 proteins on CD4 or mab T8 surfaces. When equivalent response unit (RU) amounts of gp140 proteins were captured on these two surfaces (Fig. 14A), the same level of anti-gp120 V3 mab 19b and anti-gp41 mab 2F5 binding was observed (Figs. 14B and 14C). Mab 2F5 reactivity could either be reacting with captured cleaved gp41 or binding to gp41 in uncleaved gp140. Nonetheless, the captured gp140 proteins on both of these surfaces were near identical in their reactivity with anti-gp120 and anti-gp41 mabs.

The ability of HR-2 peptides to bind to captured gp140 on mab T8 or sCD4 surfaces was tested. Binding of the HR-2 peptides showed qualitative differences in binding to mab T8 and sCD4-bound gp140. Compared to

the T8-gp140 surface (near background binding), the DP178 HR-2 peptide bound specifically to the sCD4-gp140 surface (Fig. 14D). However, there was no binding of the scrambled DP178 peptide to mab T8-gp140 or sCD4-gp140 surfaces (Fig. 14D). Similar to HR-2 peptide DP178 binding, HR-2 peptide T649Q26L showed no binding to the mab T8-gp140 surface, and marked binding to the sCD4-gp140 surface (Fig. 14F). Taken together, these results demonstrated that sCD4 induced the binding of both HR-2 peptides, DP178 and T649Q26L, to HIV 89.6 gp140.

HR-2 peptide binding to CD4-gp140 compared to CD4-gp120 complexes. Kowalski et al. have shown that mutations in gp41 HR-2 disrupt gp41-gp120 binding, and that HR-2 contains a touch point site of gp41 non-covalent interaction with gp120 (Alam et al, Nature 381:616-620 (1996)). Thus, it was of interest to compare HR-2 binding to sCD4-gp140 complexes with sCD4-gp120 complexes in SPR assays. As in experiments in Fig. 14, HIV 89.6 gp120 or gp140 proteins were captured in equivalent amounts on sCD4 immobilized surfaces. Interestingly, specific binding of HR-2 peptide was detected on both sCD4-gp140 and sCD4-gp120 surfaces (Figs. 15A and 15B). However, there was much higher binding of both DP178 and T649Q26L HR-2 peptides to the sCD4-gp140 surface compared to the sCD4-gp120 surface (Figs. 15A, 15B). To determine if

the binding of HR-2 peptides to gp120 was induced by sCD4, HR-2 binding to sCD4-gp120 was compared with HR-2 binding to gp120 proteins captured on the T8 mab surface. As shown in Fig. 15C, the binding of both HR-2 peptides DP178 and T649Q26L to gp120 was specifically induced by sCD4 since neither HR-2 peptide bound to gp120 on the T8 mab surface. No binding of scrambled DP178 to CD4-gp140 or CD4-gp120 was detected (Fig. 15D).

Finally, to assess the affinity of the binding interactions between HR-2 peptide and sCD4 triggered HIV 89.6 gp140 and gp120, the rate constants were measured and the dissociation constant (K_d) for the binding of both HR-2 peptides, DP178 and T649Q26L to sCD4-gp140 and sCD4-gp120 (Figs. 15A, 15B, and 15E). There was little difference in the kinetics of HR-2 peptides binding to sCD4-gp140 and sCD4-gp120, indicating that the higher level of binding of the HR-2 peptide to CD4-gp140 was due to induced binding to HR1-gp41 in addition to binding sites on gp120. The binding affinity of the HR-2 peptides for gp120 and gp140 were between 1.2-2.5 μ M. The HR-2 binding was stable with relatively slow off-rates ($t_{1/2}$ values in minutes, 7.7 to 10.5 min) on both sCD4-gp120 and sCD4-gp140 complexes.

Binding of HR-2 peptide to recombinant gp41. To directly determine if HR-2 peptides can

constitutively bind to purified gp41, recombinant ADA gp41 was immobilized to a sensor surface and the binding of HR-2 peptide, DP178, determined. The HR-2 peptide DP178 bound well to immobilized gp41

5 (Fig. 16A), while no binding was observed with the scrambled DP178 peptide (Fig. 16C). To compare the binding of DP178 HR-2 peptide to recombinant gp41 versus HIV 89.6 gp140-sCD4 complex, the equilibrium binding constants (K_{eq}) of HR-2 binding to both were
10 measured. While the K_{eq} of DP178 binding to recombinant gp41 was weak at 26.7 μ M (Fig. 16E), the K_{eq} of DP178 binding to sCD4-gp140 was 10 fold stronger at 1.7 μ M (Fig. 16F).

15 *Complexes of gp120-gp41 formed on a sensor surface can be induced by sCD4 to upregulate HR-2 peptide binding.* In the preparation of HIV 89.6 gp140 envelope, there is uncleaved gp140, and cleaved gp140 components of gp120 and gp41. Thus, it is possible that HR-2
20 peptide could be induced to bind uncleaved gp140 and/or could be induced to bind to cleaved gp120 and gp41. To directly determine if binding of sCD4 to gp120 that is non-covalently bound to gp41 can upregulate HR-2 peptide binding to the sCD4-gp120-gp41
25 complex, recombinant ADA gp41 was immobilized on a sensor chip, and HIV 89.6 gp120 or a gp120 sCD4 mixture was flowed over it. It was found that gp120 bound stably to gp41 (Fig. 17). When HR-2 peptide

DP178 was flowed over the gp41-gp120-sCD4 complex, binding of HR-2 was upregulated compared to HR-2 binding to gp41 or to the gp41-gp120 complex (Fig. 17). Thus, sCD4 ligation of gp120 non-covalently bound to gp41 can upregulate either gp41 or gp120, or both, to bind to HR-2.

Neutralizing Epitopes on HIV 89.6 gp140 Before and After Ligation with sCD4. The 2F5 (anti-gp41, 10 ELDKwas) (Muster et al, J. Virol. 67:6642-6647 (1993)), mab neutralizes HIV primary isolates. Prior to ligation of cleaved 89.6 gp140 with sCD4, it was found that the 2F5 gp41 epitope was exposed. Following sCD4 ligation, the 17b CCR5 binding site 15 epitope (2-4) was upregulated and the 2F5 epitope continued to be expressed.

EXAMPLE 3

Experimental Details

20 *Proteins.* Soluble CD4 was produced by Progenics, Tarrytown, NY and was provided by the Division of AIDS, NIAID, NIH. Soluble envelope gp120 (VBD-2) and gp140 (VBD-1) proteins from HIV 89.6 primary isolate were produced using recombinant vaccinia viruses and 25 purified as described (Earl et al, J. Virol. 68:3015-3026 (1994), Earl et al, J. Virol. 75:645-653 (2001)). Briefly, BS-C-1 cells in 160 cm² flasks were infected

with vBD1 (HIV 89.6 gp140) or vBD2 (gp120) viruses.

After 2h, the cells were washed in PBS and placed in serum-free OPTI-MEM media (Gibco) for 24 -26 hr. The culture medium was then harvested by centrifugation

5 and filtration (0.2µm) and Tritox-X 100 added to 0.5%.

For some experiments, culture medium was concentrated 15-30 fold and served as a source of gp140 glycoproteins (a mix of cleaved and uncleaved forms).

Lentil lectin column purified gp140 contained ~50:50

10 cleaved and uncleaved gp140. Recombinant HIV ADA gp41 protein was obtained from Immunodiagnostics Inc.

(Woburn, MA). HIV-1 BAL gp120 was produced by ABL and provided by the Division of AIDS, NIAID, NIH.

15 *Monoclonal Antibodies.* Mab A32 was obtained from James Robinson (Tulane University, New Orleans, LA) (Boots et al, AIDS Res. Hum. Res. 13:1549 (1997)). A32 mab was affinity purified from serum-free media using a Staph Protein-G column.

20 *HR-2 Peptides.* Synthetic peptides were synthesized (SynPep, Inc., Dublin, CA), and purified by reverse phase HPLC. Peptides used in this study had greater than 95% purity as determined by HPLC, and confirmed 25 to be correct by mass spectrometry. gp41 peptides DP178, YTSЛИHSLIEESQNQQEKNEQELLELDKWASLWNWF (Wild et al, Proc. Natl. Acad. Sci. USA 19:12676-12680 (1994)), and T649-Q26L, WMEWDREINNYTSЛИHSLIEESQNQLEKNEQELLEL

(Rimsky et al, J. Virol. 72:986-993 (1998), Shu et al, Biochemistry 39:1634-1642 (2000)) were derived from HIV-1 envelope gp41 from HIV 89.6 (Collman et al, J. Virol. 66:7517-7521 (1992)). As a control for HR-2 5 peptide binding, a scrambled sequence DP178 peptide was made. For immunoprecipitations and select SPR experiments, biotinylated DP178 and DP178 scrambled peptides were synthesized (SynPep, Inc.).

10 *Surface Plasmon Resonance Biosensor Measurements.* SPR biosensor measurements were determined on a BIACore 3000 (BIACore Inc., Uppsala, Sweden) instrument. Anti-gp120 mab (T8) or sCD4 (100-300 µg/ml) in 10mM Na-Acetate buffer, pH 4.5 were directly immobilized to a 15 CM5 sensor chip using a standard amine coupling protocol for protein immobilization (Alam et al, Nature 381:616-620 (1996)). A blank in-line reference surface (activated and de-activated for amine coupling) was used to subtract non-specific or bulk 20 responses. Binding of proteins and peptides (biotinylated or free DP178, T649Q26L, DP178-scrambled) was monitored in real-time at 25°C with a continuous flow of PBS (150 mM NaCl, 0.005% surfactant p20), pH 7.4 at 10-30µl/min. Analyte (proteins and 25 peptides) were removed and the sensor surfaces were regenerated following each cycle of binding by single or duplicate 5-10 µl pulses of regeneration solution (10 mM glycine-HCl, pH 2.5 or 10mM NaOH).

All analyses were performed using the non-linear fit method of O'Shannessy et al. (O'Shannessy et al, Anal. Biochem. 205:132-136 (1992)) and the BIAevaluation 3.0 software (BIAcore Inc). Rate and 5 equilibrium constants were derived from curve fitting to the Langmuir equation for a simple bimolecular interaction (A + B = AB).

10 Results

CD4-induced binding of HR-2 Peptides to HIV 89.6 gp140 Envelope Proteins. A major goal of this study was to determine if fusion-associated conformations of gp41 could be detected using SPR assays of HR-2 peptide 15 binding. It was reasoned that if HR-2 peptides can bind gp140, the gp41 coiled-coil structure must be uncoiled, such that endogenous HR-2 is not bound to HR-1. Two such HR-2 peptides were used in this study, DP178 (Wild et al, Proc. Natl. Acad. Sci. USA 20 19:12676-12680 (1994), Rimsky et al, J. Virol. 72:986-993 (1998)) and T649Q26L (Rimsky et al, J. Virol. 72:986-993 (1998), Shu et al, Biochemistry 39:1634-1642 (2000)). DP178 contains C-terminal amino acids of HR-2 (Wild et al, Proc. Natl. Acad. Sci. USA 25 19:12676-12680 (1994), (Rimsky et al, J. Virol. 72:986-993 (1998)) while T649Q26L contains more N-terminal amino acids of HR-2 (Rimsky et al, J. Virol.

72:986-993 (1998), Shu et al, Biochemistry 39:1634-1642 (2000)).

HIV envelope gp120 proteins bind to sCD4 with a relatively high affinity (Myszka et al, Proc. Natl.

5 Acad. Sci. 97:9026-9031 (2000), Collman et al, J. Virol. 66:7517-7521 (1992)). In preliminary studies, it was found that soluble HIV 89.6 gp120 protein bound strongly to immobilized sCD4, with a K_d 23nM and with an off-rate of $1.1 \times 10^{-4} \text{ s}^{-1}$. Thus, a CD4 immobilized 10 surface allowed a very stable capture of HIV envelope, and this approach has been used to assay HR-2 peptide binding to sCD4 bound HIV 89.6 envelope proteins. To create equivalent surfaces of tethered gp140 and gp120 on CM5 sensor chips, sCD4 and anti-gp120 mab T8 15 immobilized on sensor chips were used as capture surfaces. A blank chip served as an in-line reference surface for subtraction of non-specific binding and bulk responses. Mab T8 bound HIV 89.6 gp120 with an affinity of 5.6 nM. Thus, both CD4 and the mab T8 20 provided stable surfaces for anchoring HIV envelope proteins.

Since HIV 89.6 gp140 contains both cleaved and uncleaved gp140, it was important to show that gp41 was present in CD4-gp140 complexes following capture 25 of gp140 proteins on CD4 or mab T8 surfaces. When equivalent response unit (RU) amounts of gp140 proteins were captured on these two surfaces, the same level of anti-gp120 V3 mab 19b and anti-gp41 mab 2F5

binding was observed. Mab 2F5 reactivity could either be reacting with captured cleaved gp41 or binding to gp41 in uncleaved gp140. Nonetheless, the captured gp140 proteins on both of these surfaces were near 5 identical in their reactivity with anti-gp120 and anti-gp41 mabs.

The ability of HR-2 peptides to bind to captured gp140 on mab T8 or sCD4 surfaces was next tested. Binding of the HR-2 peptides showed qualitative 10 differences in binding to mab T8 and sCD4-bound gp140. Compared to the T8-gp140 surface (near background binding), the DP178 HR-2 peptide bound specifically to the sCD4-gp140 surface. However, there was no binding of the scrambled DP178 peptide to mab T8-gp140 or 15 sCD4-gp140 surfaces. Similar to HR-2 peptide DP178 binding, HR-2 peptide T649Q26L showed no binding to the mab T8-gp140 surface, and marked binding to the sCD4-gp140 surface. Taken together, these results demonstrated that sCD4 induced the binding of both HR- 20 2 peptides, DP178 and T649Q26L, to HIV 89.6 gp140.

The A32 mab has been reported to reproduce the effect of sCD4 in triggering HIV envelope to upregulated the availability of CCR5 binding site (Wyatt et al, J. Virol 69:5723 (1995)). Thus, an A32 25 mab surface was used to determine if A32 mab could mimic sCD4 to upregulate HR-2 binding to captured gp140. Similar to sCD4, HR-2 peptide binding was markedly upregulated when gp140 (a mixture of

uncleaved gp140, cleaved gp120 and cleaved g41) was captured on the A32 mab surface compared to gp140 captured on the mab T8 surface (Fig. 26). Similar results were obtained using A32 mab and gp120.

5

EXAMPLE 4

Experimental Details

Proteins. Soluble CD4 (sCD4) was produced by Progenics, Tarrytown, NY and was provided by QBI, Inc. 10 and the Division of AIDS, NIAID, NIH. Soluble envelope (Env) 89.6 gp120 and III_B gp120 from HIV-1 89.6 and HIV-1 III_B isolates respectively, were produced using recombinant vaccinia viruses and purified as described (Baik et al, Virology 15 259(2):267-273 (1999), Center et al, J. Virol. 74(10):4448-4455 (2000), Earl et al, J. Virol. 68(5):3015-3026 (1994)). Briefly, BS-C-1 cells in 160 cm² flasks were infected with vBD2 or vPE50 recombinant vaccinia viruses. After 2h, the cells 20 were washed in PBS and placed in serum-free OPTI-MEM media (Gibco) for 24 -36 hr. The culture medium was harvested by centrifugation and filtration (0.2μm) and Triton-X 100 added to 0.5%. HIV-1 BaL and JRFL gp120 25 proteins were produced by ABL and provided by QBI, Inc. and Division of AIDS, NIAID, NIH.

Monoclonal Antibodies. Human mAbs against a conformational determinant on gp120 (A32), the gp120 V3 loop (mAb 19b), and the HIV-1 coreceptor binding site mAbs, 17b and 48d were produced and used as 5 described (Scearce and Eisenbarth, Methods in Enzymology 103:459-469 (1983)). 2G12 mAb was obtained from the AIDS Reference Repository, NIAID, NIH. T8 is a murine mAb that maps to the gp120 C2 region and reacts with many HIV-1 envelopes including HIV-1 89.6. 10 T8 was a gift from P. Earl (Laboratory of Viral Diseases, NIH, Bethesda, MD).

Peptides. Peptides were synthesized (SynPep, Inc., Dublin, CA), and purified by reverse phase HPLC. 15 Peptides used in this study had greater than 95% purity as determined by HPLC, and confirmed to be correct by mass spectrometry. HR-2 gp41 peptide DP178, YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF, and DP107, NNLLRAIEAQHQHLLQLTVWGIKQLQARILAVERYLKDQ were 20 derived from HIV-1 89.6 envelope gp41 HR-2 and HR-1 regions respectively (Collman et al, J. Virol. 66(12):7517-7521 (1992), Wild, Proc. Natl. Acad. Sci. 91:9770-9774 (1994)). As a control for HR-2 peptide binding, randomly scrambled sequences of DP178 25 (scrDP178) and DP107 (scrDP107) peptides were also made. For precipitations and surface plasmon resonance (SPR) experiments using the streptavidin chip, biotinylated DP178 and scrDP178 peptides were

synthesized (SynPep, Inc.). The following C4 and V3 peptides were used in the peptide blocking experiment-
V3_{89.6P} - TRPNNNTRERLSIGPGRAFYARR; C4- IKQIINMWQKVKGAMYAPPIS;
C4-V3_{MN} - KQIINMWQEVGKAMYACTRPNYNKRKRIHIGPGRAFYTTK; and
5 C4-V3_{89.6P} - KQIINMWQEVGKAMYATRPNNNTRERLSIGPGRAFYARR
A scrambled amino acid version of the V3 component of C4-V3 (C4-scrV3) was also synthesized as a control peptide.

10 *Surface Plasmon Resonance Biosensor Measurements.*
SPR biosensor measurements were determined on a BIAcore 3000 (BIAcore Inc., Uppsala, Sweden) instrument and data analysis was performed using BIAevaluation 3.0 software (BIAcore Inc.). For the 15 "capture assay", anti-gp120 mAb (T8, A32) or sCD4 (100-300 µg/ml) in 10mM Na-Acetate buffer, pH 4.5 were directly immobilized to a CM5 sensor chip using a standard amine coupling protocol for protein immobilization (Alam et al, Nature 381:616-620 20 (1996)). A blank in-line reference surface (activated and de-activated for amine coupling) was used to subtract non-specific or bulk responses. Binding of proteins and peptides (biotinylated or free DP178/T-20, scrDP178) was monitored at 25°C with a continuous 25 flow of PBS (150 mM NaCl, 0.005% surfactant p20), pH 7.4 at 10-30µl/min. Analyte (proteins and peptides) were removed and the sensor surfaces were regenerated by single or duplicate 5-10 µl pulses of regeneration

solution (10 mM glycine-HCl, pH 2.5 or 10mM NaOH). For determination of HR-2 peptide specific binding, it was critical to use freshly prepared peptides prior to each experiment in order to minimize background binding to CD4 surfaces. Additionally, non-specific binding of HR-2 peptides to capture surfaces (CD4 or mAb T8) was subtracted to determine specific binding of HR-2 peptides to gp120 envelope proteins (Fig 27). Antibody blocking experiments were performed by mixing gp120 with excess (3-5 fold) of mAbs and pre-incubating at RT for 20 min. These mixtures were then injected for binding studies as described above.

For the "streptavidin (SA)-chip assay", 200-300 RU of the HR-2 peptide DP178, scrambled DP178 (scrDP178) and scrambled DP107 (scrDP107) peptides were bound to individual flow chambers of a SA-chip. The scrDP107 surface was used as a reference surface for subtracting bulk and non-specific binding. Soluble HIV-1 Env proteins (89.6 gp120, III_B gp120) at 100-200 μ g/ml were pre-incubated with 3-5 molar excess of various mAbs (A32, 2G12, 17b, 48d, 19b) or sCD4 for 30 min at RT. Soluble gp120 proteins, mAbs or gp120 proteins pre-incubated with mAbs were then injected at 20 μ l/min for 2-3 min over each of the peptide-SA surface. Binding data was acquired following in-line reference subtraction of binding from the scrDP107 (HR-1) surface.

Precipitation of HIV-1 Envelope Proteins and Western Blot Analysis. Soluble HIV-1 gp120 proteins (40 μ g) were incubated for 1h with or without recombinant sCD4 (14 μ g) or mAb A32 (80 μ g), and a dose range (0.5 to 12.5 μ g) of either biotinylated DP178/T-20 or biotinylated scrDP178 as a control in a total volume of 50 μ l PBS for 1h followed by incubation (4h) with 50 μ l suspension of streptavidin-agarose beads (Sigma Chemicals, St. Louis, MO). DP178-gp120 complexes were washed x3 with 500 μ l of PBS, resuspended in SDS-PAGE sample buffer containing 2-ME, boiled for 5 min, and loaded onto SDS-PAGE on 4-20% polyacrylamide gels. Gels were transferred to immunoblot membranes for Western blot analysis with mAb T8 (anti-gp120 C2 region). All statistical analyses performed using the GraphPad InStat software and the paired t-test.

Results

Inducible binding of HR-2 peptide to soluble HIV-1 Env gp120. In initial experiments, when 89.6 gp120 was covalently immobilized on a BIAcore CM5 sensor chip, it was observed that in comparison to scrambled DP178 (scrDP178) and in the presence of sCD4, the HR-2 peptide DP178 bound specifically to 89.6 Env gp120 proteins. These data suggested that there might be an HR-2 binding site on Env gp120. However, this was not a preferred protocol since random covalent coupling

could lead to heterogeneity in the immobilized envelope protein and may also cause alterations in protein conformation. In addition, since the goal was to determine whether the gp120 HR-2 binding site was 5 inducible by sCD4 or A32 mAb, the decision was made not to employ a binding assay that required direct immobilization of Env proteins. Instead, the binding of HR-2 peptide DP178 to soluble HIV-1 Env 89.6 gp120 was monitored using two different BIACore binding 10 assays, termed the "capture assay" and the "SA (streptavidin)-chip assay".

In the "capture assay", described in Fig 27, DP178 binding to gp120 was assayed on 3 different capture surfaces- mAb A32, CD4 and mAb T8, each of 15 which were immobilized on individual flow cells of the same sensor chip. HIV-1 Env 89.6 gp120 was bound to equivalent levels on each of these surfaces (as judged by their bound RU and reactivity with the anti-V3 loop mAb, 19b) and then after a brief period of 20 stabilization, DP178 or scrDP178 peptide was injected over them (Fig 27A). The binding of DP178 peptide over each of the capture mAb or CD4 surfaces was also monitored (Fig 27B), and this non-specific binding was subtracted from the binding signal derived from the 25 interaction of DP178 or scrDP178 with gp120 bound surfaces (Fig 27C). Using this binding assay, specific induction of DP178 binding was observed only when gp120 was bound to CD4 or A32 but not when bound to T8

(Fig. 27D). The scrambled sequence of DP178 (scrDP178) gave a low level of binding to each surface (Fig 27E) and this low level of binding of scrDP178 to gp120 suggested that some degree of electrostatic interactions might be involved. In contrast, there was a 3 to 4 fold increase in the binding of DP178 to gp120 bound to either CD4 or A32. Thus, a specific induction of DP178 binding to gp120 was clearly observed after the Env protein was bound to either 5 sCD4 or mAb A32. As a control, when gp120 was bound to mAb T8, no differences were observed between binding 10 of DP178 and scrDP178 peptide gp120 (Fig. 27D). Although an alternative explanation of the binding data shown in Fig. 27D could be that DP178 binding was 15 inhibited by mAb T8, this was ruled out since DP178 did not bind to gp120 alone or when preincubated with another anti-gp120 mAb, 2G12 (described in Fig 28 below). Thus the lack of binding of DP178 to gp120 bound to T8 was not due to inhibition, but rather was 20 due to lack of induction of DP178 binding by mAb T8. Moreover, the induction of DP178 binding to gp120 after sCD4 or A32 binding was specific for the HR-2 peptide, since no binding was observed with the HR-1 peptide, DP107 to gp120 on A32 surfaces (Fig 27F).

25 The affinity of DP178-gp120 binding was measured to be 820nM. Due to the biphasic nature of the binding, both a faster (0.023 s^{-1}) and a relatively slower component of the dissociation phase (0.001 s^{-1})

were obtained. The latter component was predominant and corresponded to a relatively long half-life ($t_{1/2}$) of 11.5 minutes.

Next, a determination was made as to whether 5 gp120 coreceptor usage was a determinant for HR-2 binding and HIV-1 III_B gp120 (CXCR4) and HIV-1 BaL and HIV-1 JRFL gp120 (CCR5) were tested. DP178 binding was only observed with Env gp120 from the dual tropic HIV-1 isolate, 89.6, and the CXCR4 dependent isolates 10 (III_B gp120, Fig 31C), but not with gp120 from CCR5-utilizing isolates, BaL (Fig 27) and JRFL. As shown in Fig 27G and 27H, neither DP178 nor scrDP178 bound to BaL gp120 complexed with A32, CD4 or T8. Thus, the induction of DP178 binding to gp120 was only observed 15 with Env proteins that were derived from viruses that utilized the coreceptor CXCR4.

The above results were next tested by reversing the orientation of the reactants. In these experiments (the "SA-chip assay"), biotinylated HR-2 peptides 20 DP178 and scrDP178 were immobilized on adjacent flow cells of a streptavidin sensor chip (SA chip). Using biotin-scrDP107 as an additional negative control, the binding of mAb A32, 89.6 gp120 or a mixture of the 89.6 gp120 and saturating amounts of mAb A32 was 25 assayed over both DP178 and scrDP178 surfaces (Figure 28). The non-specific and bulk effect was measured over scrDP107 surface and this surface was used for automated in-line reference subtraction. On the

scrDP178 surface, an extremely low level of background signal (10-20 RU) was observed when either mAb A32 or 89.6 gp120 was injected (Fig 28B). This was also true when mAb A32 was flowed over the DP178 surface (Fig 5 28A).

However, a low level of constitutive binding (about 50 RU) above the background was detected when 89.6 gp120 was injected over DP178 or scrDP178 surfaces (Fig. 28A). More importantly, compared to 10 constitutive binding of DP178, there was significant induction of binding signal (about 8-10 fold) when 89.6gp120 protein, pre-incubated with A32 mAb was injected over DP178 (Fig 28A, $p<0.02$ for +A32 vs -A32; $n= 3$). This induction was clearly much larger than 15 what would be attributed simply to an increase in mass of A32-gp120 complex when compared to gp120 alone. In the experiment shown in Fig 28A, 44RU of gp120 binding to DP178 would correspond to a maximum of 132 RU of binding of gp120-A32 complex, if binding was simply 20 due to mass effect. However, ~ 400 RU (3-fold higher than expected due to mass effect only) of binding of A32-gp120 to DP178 (Fig 28A) was observed. In contrast, the increase in signal from A32- gp120 binding to scrDP178 (<200 RU) was predominantly due to 25 mass effect (compare +A32 curves in Fig 28A and 28B). Moreover, data presented in Fig. 27 clearly showed that A32 mAb and CD4 induced markedly higher binding of DP178 to gp120. Taken together, the data shown in

Figures 27 and 28 demonstrated that there is a specific DP178 binding site on HIV-1 89.6 Env gp120 protein, and that this binding is induced by sCD4 and A32 mAb.

5 Both the "capture assay" and the "SA-chip assay" allowed the detection of a discernable and significant difference between induced and constitutive binding of DP178. Interestingly, the binding of A32-induced gp120 to DP178 appeared to be bi-phasic and could be
10 resolved into two components based on the dissociation rates (off-rate) - a relatively faster off-rate of 0.020 s^{-1} and a much slower rate of 0.0016 s^{-1} (Fig 28D). On the other hand, the measured off-rate on the scrDP178 surface appeared to have a single component
15 and was similar to the faster off-rate (0.017 s^{-1}) observed on DP178 surface. In fact, subtraction of scrDP178 binding signal from DP178 revealed only the slower kinetic component associated with A32 induced binding to DP178 (compare curves with solid line and
20 solid circle in Fig. 28C). These differences in the off-rates, therefore, suggested that mAb A32 induced HR-2 binding involved both a sequence independent (fast off-rate) and a sequence-dependent (slow off-rate) binding to Env gp120. Thus, it is likely that
25 there is both an electrostatic (faster off-rate) and a conformational component (slower off-rate) involved with induced binding of DP178 to HIV-1 89.6 gp120.

Together, these components contributed to the biphasic nature of the observed binding interactions.

Precipitation of Env gp120 protein by DP178. To further confirm the finding that the HR-2 peptide DP178 has an inducible binding site on gp120, and to study binding interactions of HR-2 to gp120 in solution, biotinylated DP178 and scrDP178 peptides were used to bind to 89.6 gp120 in solution in the presence or absence of A32 mAb or sCD4. The bound envelope proteins were precipitated using streptavidin-agarose beads and then analyzed by Western blot analysis using T8 mAb. A representative blot of three performed is shown in Fig 29A and 29B for A32 mAb and sCD4, respectively.

In the absence of sCD4 or mAb A32, a slightly higher amount of 89.6 gp120 protein was precipitated with DP178 when compared to those with scrDP178 (for blot 29A, band density for lanes 3 and 4 were 1.0 and 0.6 ODunits/mm² respectively). The same was true for bands shown in lanes 3 and 4 in Fig 29B. These data confirmed that there is a low level of constitutive binding of DP178 to gp120.

When the precipitations were carried out in the presence of mAb A32 or sCD4, significant differences in DP178 binding were observed when compared to those observed in its absence. In the presence of A32 mAb, the means of the density of the bands in lane 3 were

significantly higher than those in their absence (Figs. 29A and 29C, * p<0.005, lane 1 versus lane 3). This was also true when precipitates obtained in the presence of sCD4 were compared to those in their 5 absence (Figs. 29B and 29C, ** p<0.001, lane 1 versus 3). Therefore, the HR-2 peptide precipitation studies confirmed the observations using BIAcore assays that both A32 mAb and sCD4 induced enhanced binding of HR-2 peptide DP178 to soluble gp120 Env proteins.

10

Induction and blocking of HR-2 peptide binding. Using the capture surfaces of sCD4 and mAbs A32 and T8, an association was observed between induction of 17b binding (coreceptor binding site) and DP178 15 binding to gp120. Thus, both sCD4 and mAb A32, which triggered up-regulation of 17b binding, also induced binding of DP178, while mAb T8 induced neither (Figs. 27-29). Therefore, it was of interest to determine whether binding of 17b and other anti-gp120 mAbs would 20 have any effect on A32 induced binding of DP178 to gp120. First, the decision was made to confirm the specificity of mAb A32 induced binding of DP178 by testing whether other anti-gp120 mAbs would also have a similar effect. Unlike some Env proteins (e.g., BaL 25 gp120), 89.6 gp120 bound constitutively to 17b mAb even in the absence of sCD4 or A32 triggering. Thus, using the 'SA-chip assay' and 89.6 gp120 pre-incubated with saturating concentrations of 17b mab or 2G12 mab

(a human neutralizing mab Ab), it was possible to test the effect of these mAbs on HR-2 peptide binding to gp120. In contrast to the observations with mAb A32, neither 2G12 nor 17b mAb induced any enhancement of 5 the binding of gp120 to DP178 (Fig. 30A and 30B).

While no differences were observed on DP178 binding in the presence or absence of mAb 2G12, addition of mAb 17b gave a binding signal lower than those observed with gp120 alone (Fig 30B). This suggested that mAb 10 17b might have an inhibitory effect on DP178 binding. Interestingly, Zhang et al had reported that binding of 17b mAb to gp120 weakened the subsequent binding of sCD4 to gp120.

In order to determine whether 17b mAb would have 15 an inhibitory effect on A32-induced DP178 binding to gp120, 89.6 gp120 was first pre-incubated with a saturating concentration of mAb A32 and then added a saturating concentration of mAb 17b. This mixture was then injected over a SA-biotinylated DP178 surface. As 20 shown in Fig 30C, the addition of saturating amounts of 17b to gp120 preincubated with A32 caused complete inhibition of the binding of gp120 to DP178. This inhibitory effect showed that 17b mAb could either reverse the changes induced by mAb A32 on gp120 or 25 directly inhibit the binding of DP178 to gp120. Furthermore, 17b mAb pre-bound to gp120, which was subsequently captured on mAb A32 surface ("capture assay"), could also block the induction of binding of

DP178. As shown in Fig 30D, a dose-dependent inhibitory effect of 17b Fab on DP178 binding to gp120 captured on A32-immobilized surface was observed. Since both 17b mAb and 17b Fab could cause complete 5 blocking of DP178 binding, this effect was not due to steric hindrance. To test the specificity of 17b blocking, several other mAbs were used - an HIV-1 neutralizing human mAb (2G12), an anti-V3 mAb (19b), and a 17b-like mAb, 48d (Fig 30E, 30F). ~80% blocking 10 with 48d (and 17b) was observed, and partial blocking of HR-2 peptide binding to gp120 (mean blocking = 68%, P<0.02, n=3) with the anti-V3 loop antibody, 19b was observed. In contrast, pre-incubation with mAb 2G12, induced no significant change in RU binding of DP178 15 (n=3, P=ns). The relative blocking effect of each of these mAbs is summarized in Fig 30F. Taken together, these Ab blocking studies suggested that the A32-inducible DP178 binding epitope probably lies close to the HIV-1 coreceptor binding site on CXCR4-utilizing 20 gp120s and the V3 loop. However, it is possible that conformational changes induced by the binding of 17b-like antibodies may alter the conformation of the DP178 binding site, much like the affect they have on the CD4 binding domain (Wyatt et al, Nature 25 393 (6686) :705-710 (1998)).

Binding of DP178 to gp120 is inhibited by HIV-1 gp120 C4 peptides. The gp120 C4 region is centrally

located within the CCR5 binding site in the β 20- β 21 strands of the gp120 bridging sheet (Wyatt et al, J. Virol. 69:5723-5733 (1995)). To directly map the binding site of HR-2 peptide DP178 on gp120, a determination was made as to whether peptides containing the C4 region or the V3 loop could block the binding of HR-2 to A32-gp120 complexes. It was found that both C4-V3 and C4-scrV3 peptides significantly blocked the binding of DP178 to gp120 (Fig. 31, mean % blocking was 85 and 63 respectively; $p<0.005$ and $p<0.02$ for C4-V3 and C4-V3scr peptides respectively, $n=3$), demonstrating that the blocking peptide contained C4 sequences. Addition of either C4-V3_{89.6} or C4-V3_{MN} peptides resulted in strong blocking of HR-2 peptide binding (Figures 31A and 31B). Although relatively weaker than C4-V3 sequences, preincubation with a shorter C4 sequence alone also resulted in significant blocking (Fig 31, 27% blocking, $n=3$, $p<0.02$). In contrast, no significant blocking was observed with the V3 peptide ($n=3$, $p=ns$). Taken together, these data demonstrated that the C4 region significantly blocked the binding of HR-2 to HIV-1 gp120.

25 Conclusions

The data described above demonstrate that sCD4 and mab A32 induce the binding of the DP178/T-20 peptide to the CXCR4 binding site region of gp120. In

1987, Kowalski et al. demonstrated that insertional mutations in gp120 in the HR-2 region disrupt gp120-gp41 and suggested the gp41 HR-2 region to be a "touchpoint" for gp41-gp120 interactions (Kowalski et al, Science 237(4820):1351-1355 (1987)). However to date, significant binding of HR-2 directly to gp120 has not been demonstrated. Derdeyn and colleagues have recently suggested that co-receptor usage is an important determinant of HIV-1 resistance to the fusion inhibitor HR-2 peptide, DP178 or T-20 (Derdeyn et al, J. Virol. 74(18):8358-8367 (2000), Derdeyn et al, J. Virol. 75(18):8605-8614 (2001)). Interestingly, the dependence on V3 sequences was independent of mutations that occurred in the HR-1 region of gp41 (Derdeyn et al, J. Virol. 74(18):8358-8367 (2000)). This V3/co-receptor mediated T-20 resistance was in part due to V3 mediated changes in the viral entry rate with T-20 resistance in the presence of faster viral entry (Reeves et al, Proc. Natl. Acad. Sci. USA 99(25):16249-16254 (2002)). In addition, V3 mutations could affect the ability of T-20 to inhibit fusion by modulating the interactions of gp120 with the HR-2 peptide itself. The studies described above addressed the question of whether HR-2 peptide DP178/T20 can bind to gp120.

The data presented above are of interest for several reasons. First, these data represent a novel measurable manifestation of conformational changes

that can be induced in solution on gp120 by sCD4 and mAb A32. Second, these data raise the notion that native gp41 HR-2 may interact with gp120 following sCD4 ligation of gp120 during gp120 receptor-mediated activation. Third, the results provide an additional potential mechanism of V3 sequence modulation of T-20 resistance, that of modulation of coreceptor binding site interactions with either gp41 HR-2 or with T-20. Finally, the data bear the design of HIV vaccine 10 immunogens with "constrained" envelope conformations.

The HXBc2 core variable loop deleted envelope protein contains the C4 region, binds C4, yet did not contain V3, nor was able to bind the HR-2 peptide, DP178. Thus, although the binding site of DP178 or 15 gp120 is at or near the C4 region, the gp120 variable loops are required for HR-2 binding to gp120.

One binding component of DP178/T20 interaction with gp120 had fast dissociation kinetics, was sequence independent, and likely was electrostatic in 20 nature. However, there was clearly an additional component of DP178 binding to gp120 induced by sCD4 that displayed slower dissociation kinetics. Induced DP178 binding to gp120 was demonstrated both using surface plasmon resonance binding assays and using an 25 assay of biotinylated DP178 precipitation of gp120. From the C4 peptide blocking data, it was hypothesized that induced HR-2 peptide binding represented sCD4-induced changes in the C4 region.

It should be pointed out that it remains unknown if the native gp41 HR-2 in the context of HIV-1 virion is able to directly interact with gp120 during gp41-mediated fusion. If this HR-2 gp120 interaction is 5 relevant to normal function of the HIV-1 envelope during receptor mediated envelope activation, it would be expected that C4 peptides should be able to modify and potentially reverse the DP178-mediated inhibition of HIV-induced fusion mediated. Thus, one unifying 10 hypothesis to explain the biological relevance of the observations would be that native gp41 HR-2 interacts with a moderate affinity binding site on gp120 both before and after CD4 binding as a transient pre-fusion competent envelope conformation on CXCR4 utilizing 15 HIV-1 Env. Once this interaction is displaced by co-receptor binding to gp120, the Env assumes a fusion competent conformation and the high affinity HR-2/HR-1 interaction occurs in the context of cell fusion. In this hypothetical model, one would predict the HR-2 20 interaction with gp120 would be of lower affinity than that of HR-2 interaction with gp41 HR-1.

Interestingly, it has been found that high concentrations of C4 peptide (50-200 μ g/ml) can reverse DP178 inhibition of HIV-1 induced syncytia formation. 25 Thus, the observations of HR-2 binding to gp120 *in vitro* also may be relevant to HIV-1 envelope function in native virions.

Finally, one strategy for design of HIV vaccine immunogens is to produce "constrained" HIV Env proteins with exposed immunogenic gp120 epitopes. One strategy to produce "constrained" gp120 envelope structures would be to stabilize the coiled-coil region of gp41 in an "open" position using DP178/T20 HR-2 peptide bound to HR-1. However, the data in this study demonstrate that in addition to binding to gp41, the HR-2 peptide can bind to CXCR4-utilizing gp120s as well. It is contemplated that "constrained" gp120-HR-2 peptide complexes can induce broadly reactive neutralizing antibodies when compared to the repertoire of antibodies induced by gp120 alone. (See also Alam et al, AIDS Research and Human Retroviruses, 20:836-845 (2004)).

* * *

All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. An isolated immunogen comprising an HIV envelope protein bound to a ligand, which ligand upregulates at least the CD4 binding site on said protein,

wherein said protein comprises gp120, and
wherein said immunogen further comprises an HR-2 peptide directly bound to gp120 at a CD4 inducible site.

2. The immunogen according to claim 1 wherein said protein, said ligand and said HR-2 peptide are crosslinked.

3. The immunogen according to claim 1 wherein said protein is gp120 or uncleaved gp140.

4. The immunogen according to claim 1 wherein said ligand is an antibody, or Fab₂ or Fab fragment thereof.

5. The immunogen according to claim 1 wherein said ligand binds to a site on gp120 to which mab A32 binds.

6. The immunogen according to claim 1 wherein said ligand is mab A32, or Fab₂ or Fab fragment thereof, or mimic thereof.

7. The immunogen according to claim 1 wherein said protein is in soluble form.

8. The immunogen according to claim 1 wherein said protein is associated with a cell vesicle or liposome.

9. The immunogen according to claim 1 wherein said HR-2 peptide is DP178 or T649Q26L.

10. A composition comprising at least one immunogen according to claim 1 and a carrier.

11. A method of inducing the production of neutralizing antibodies to HIV in a mammal comprising administering to said mammal an amount of said immunogen according to claim 1 sufficient to effect said induction.

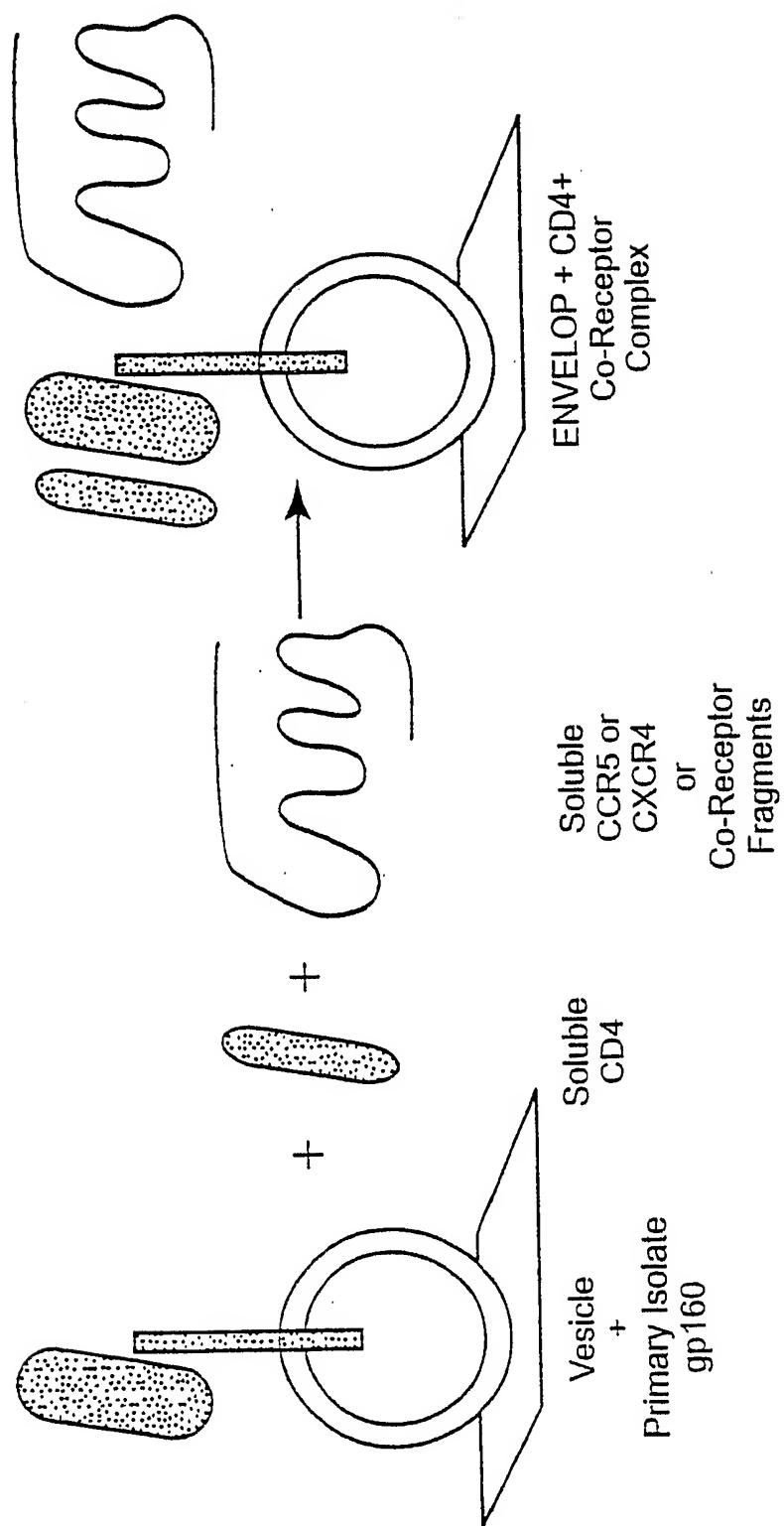


FIGURE 1

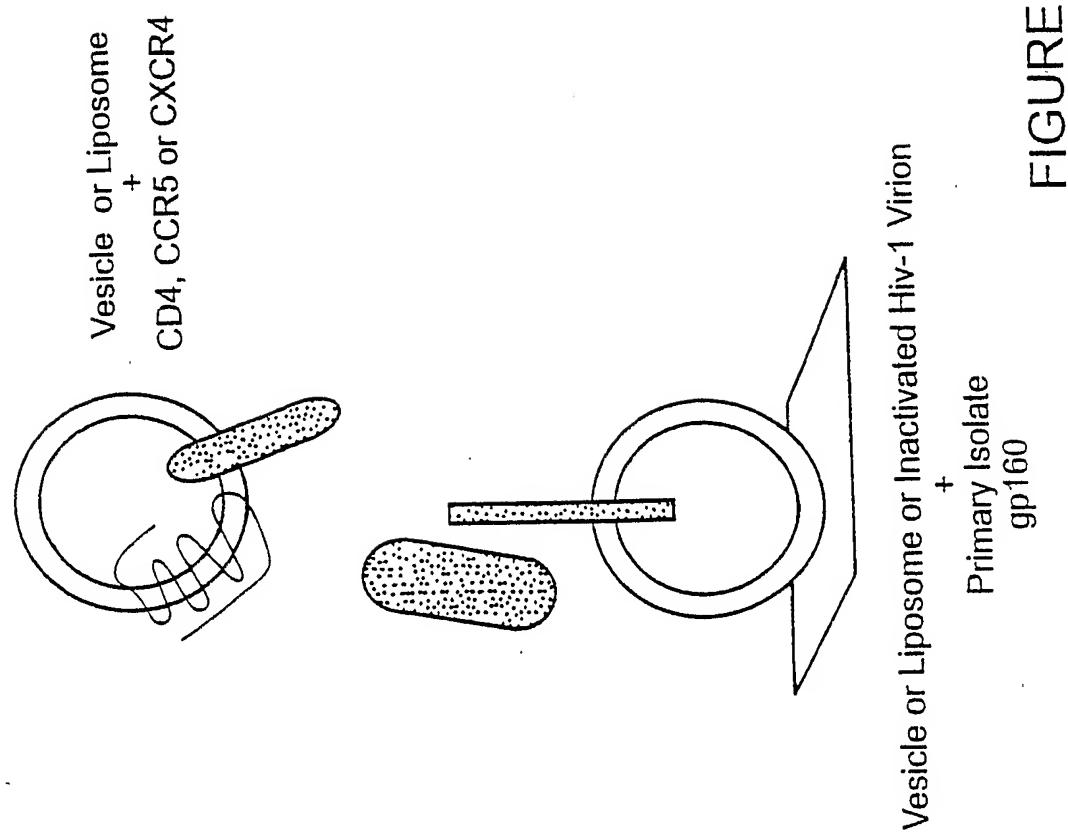
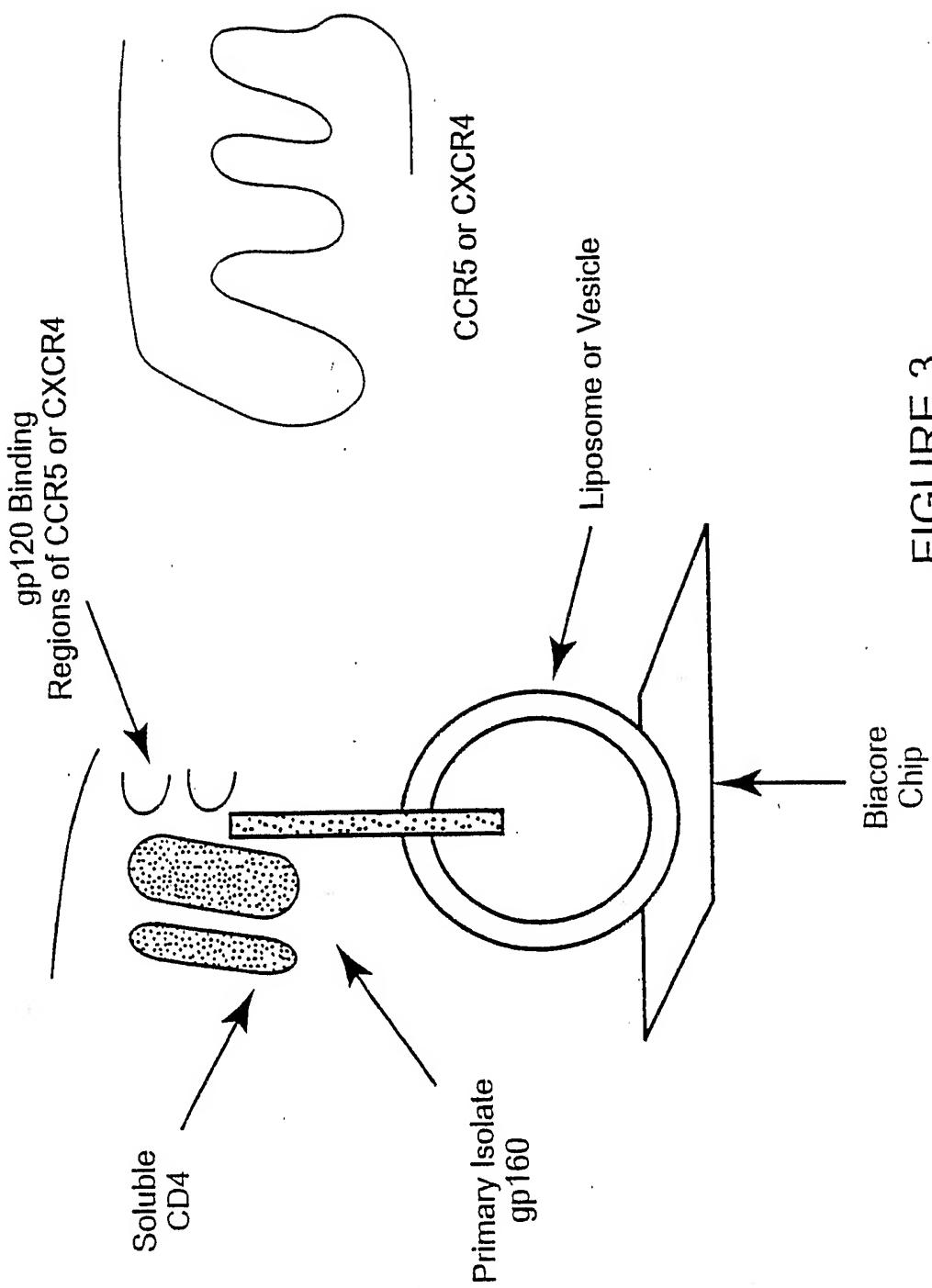


FIGURE 2



BiAcore measurements of binding interactions
between soluble gp120 (DH12) and CD4

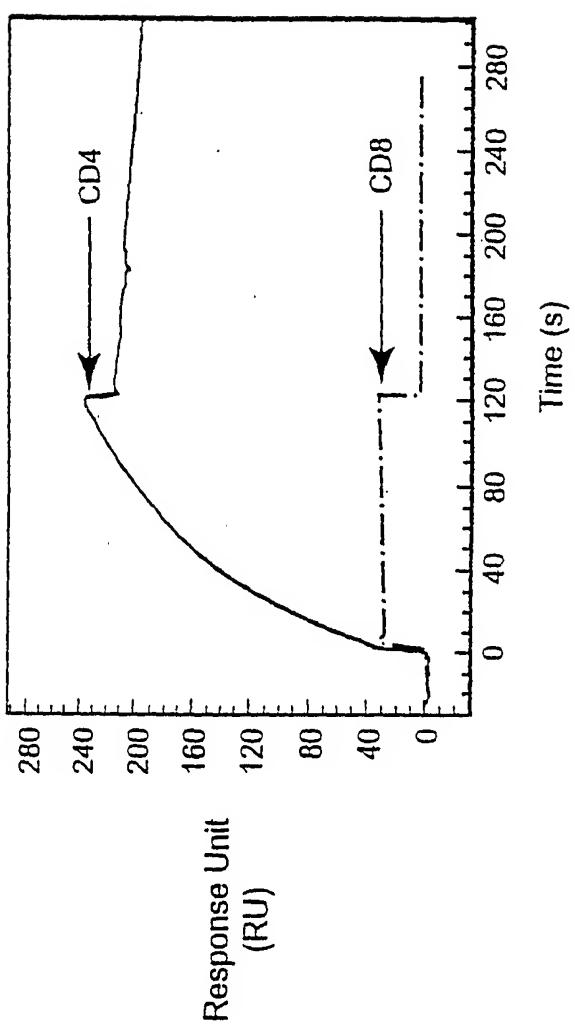


FIGURE 4

Binding interactions between soluble gp120 proteins and CD4.

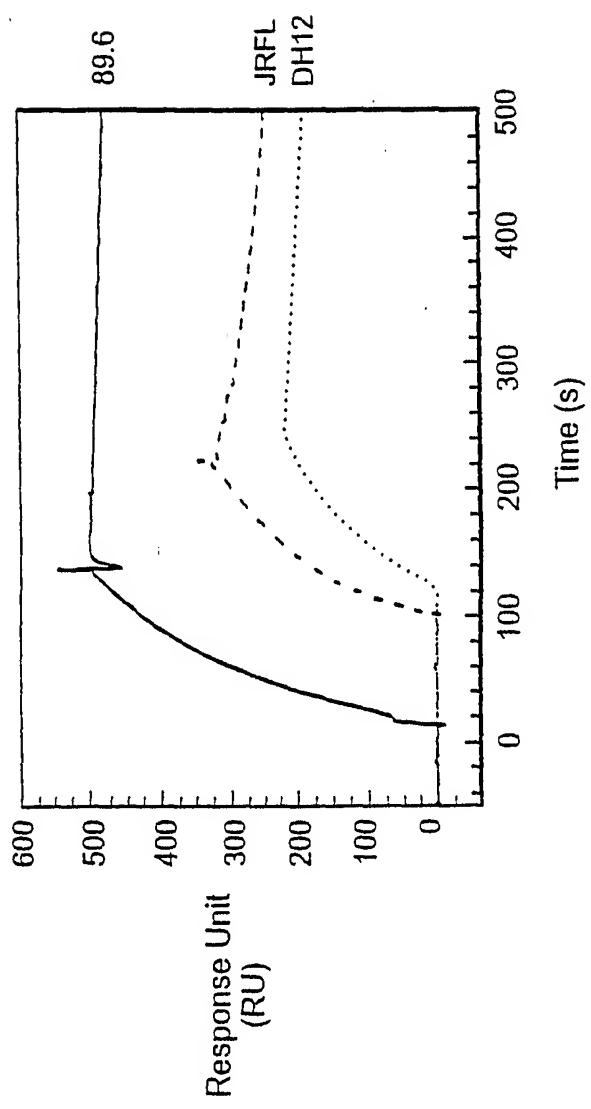


FIGURE 5

Kinetics and binding affinities of the interactions
between gp120 and CD4

gp120	$k_a M^{-1} s^{-1}$ (on-rate)	$k_d s^{-1}$ (off-rate)	K_d M	$t_{1/2}$ min
JRFL	6.0×10^3	8.84×10^{-4}	1.5×10^{-7}	14
DH12	4.9×10^3	3.86×10^{-4}	7.8×10^{-8}	30
89.6	4.6×10^3	8.48×10^{-5}	1.8×10^{-8}	136

FIGURE 6

Binding of soluble CD4 to gp120-expressing membranes from CHO-wt cells

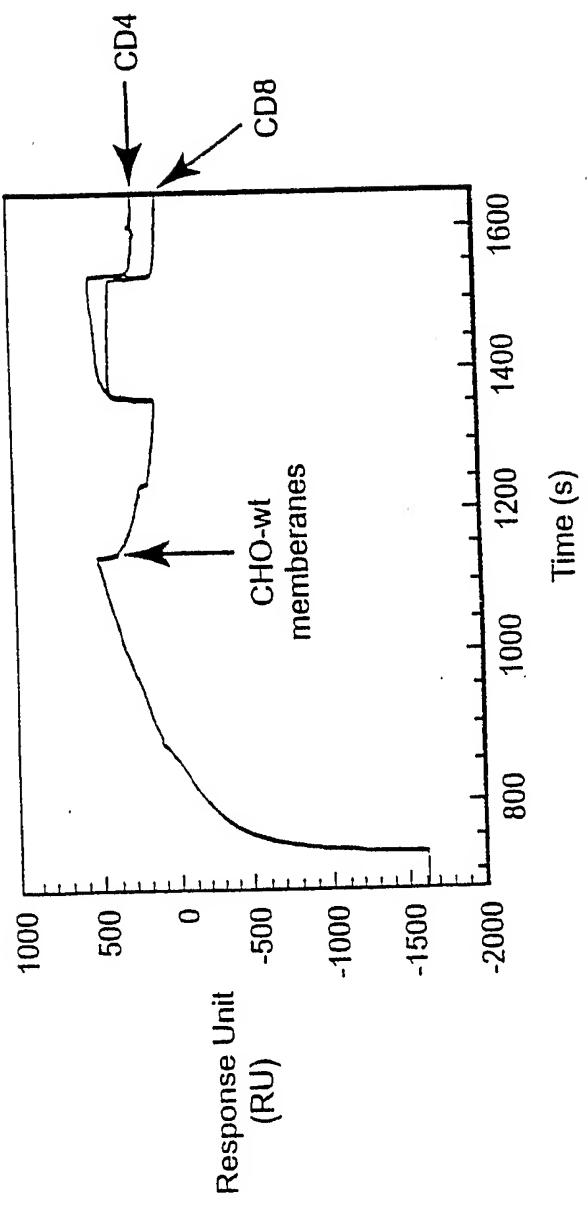


FIGURE 7

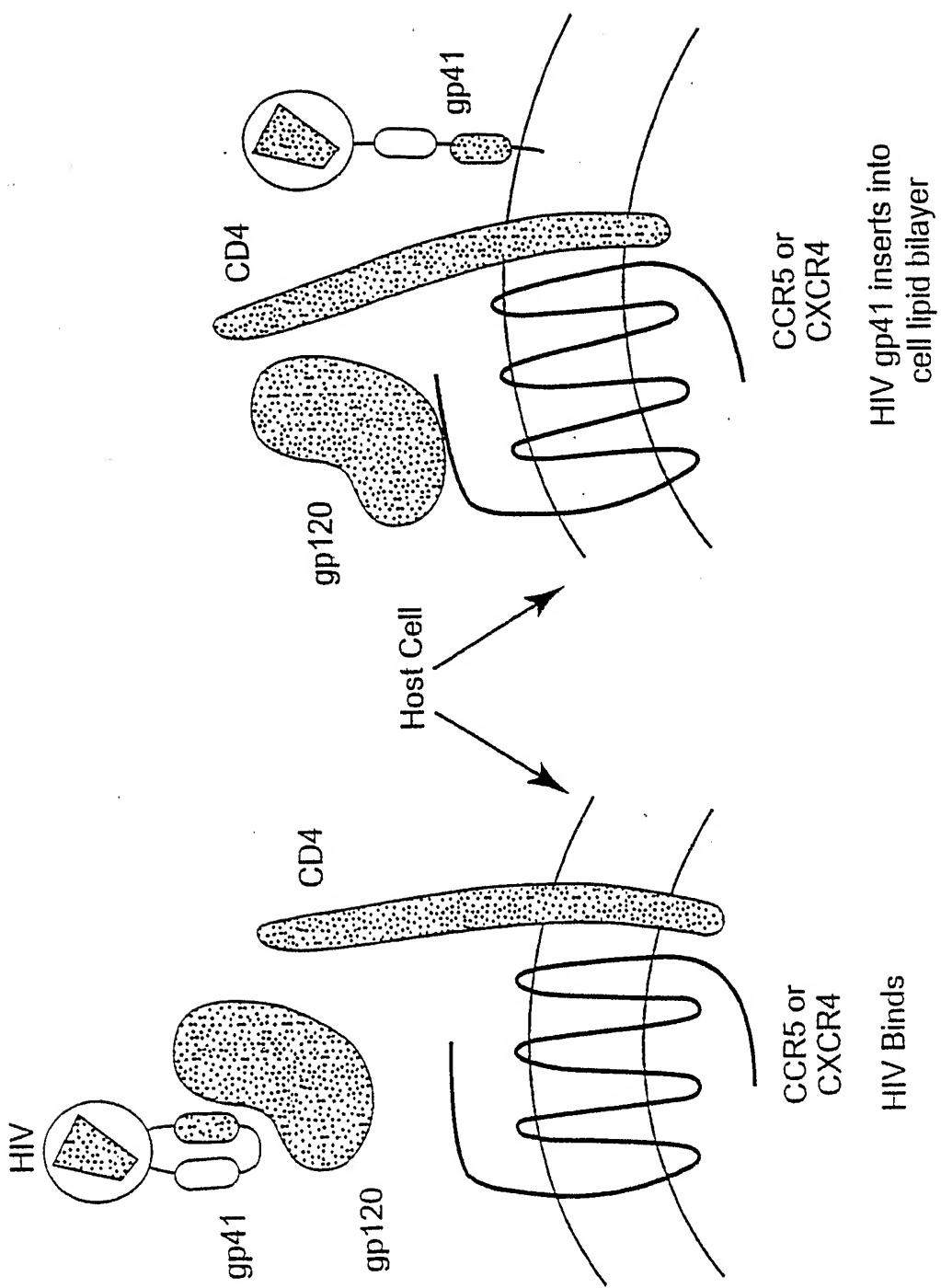


FIGURE 8

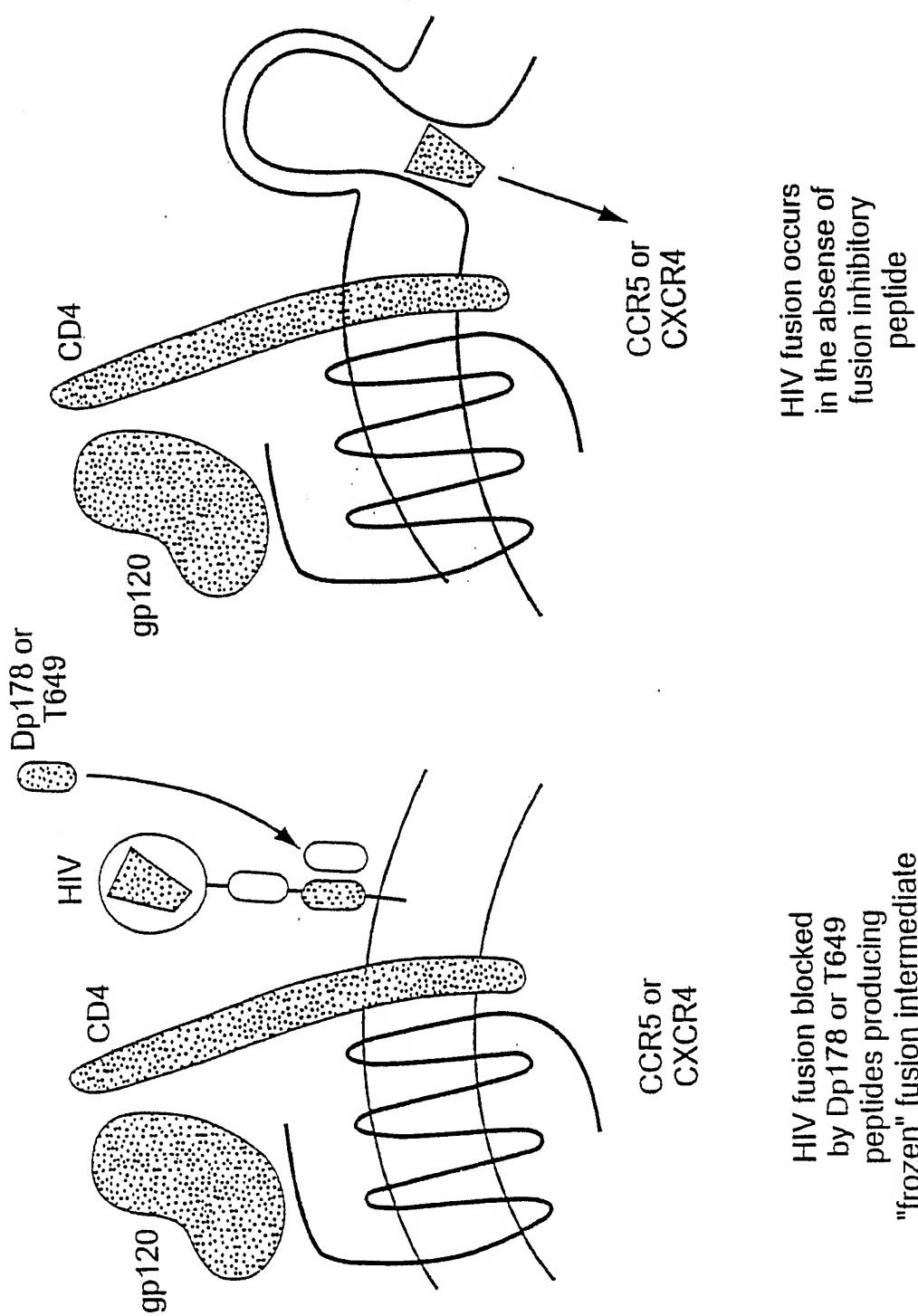


FIGURE 9

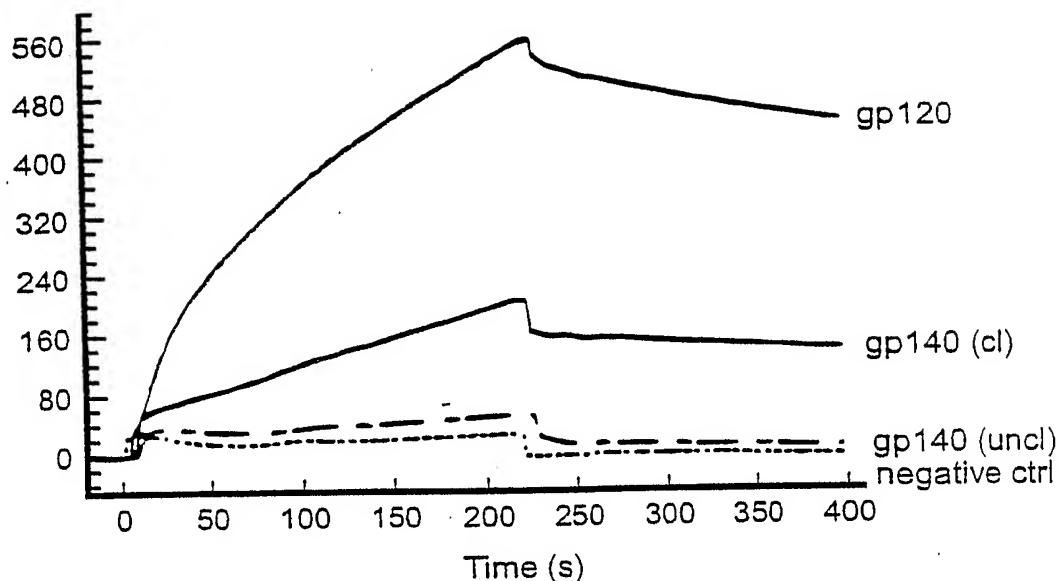


FIGURE 10A

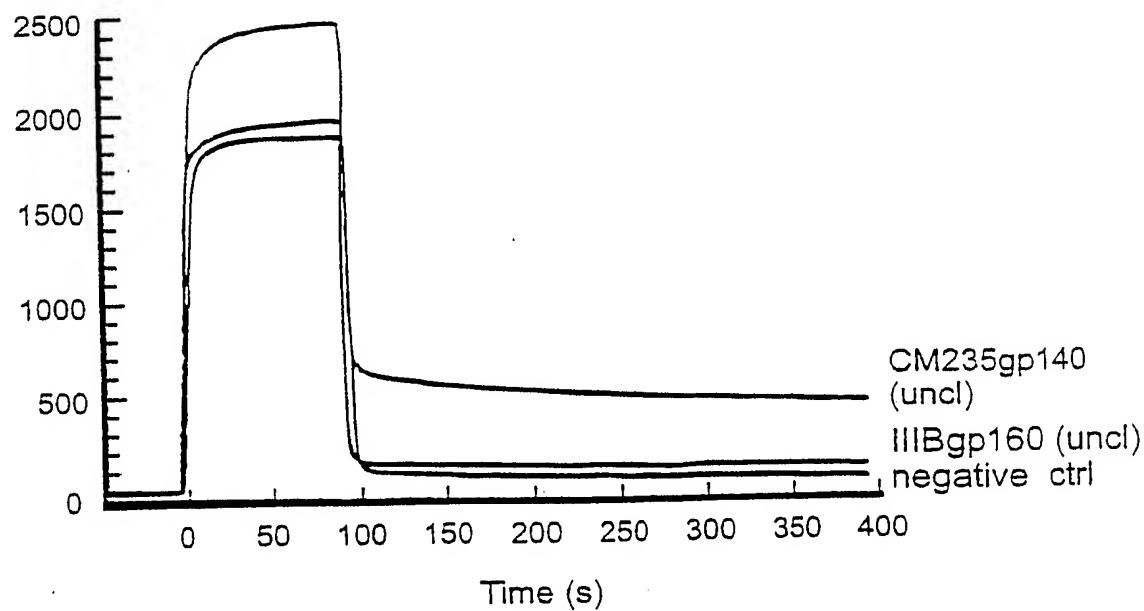


FIGURE 10B

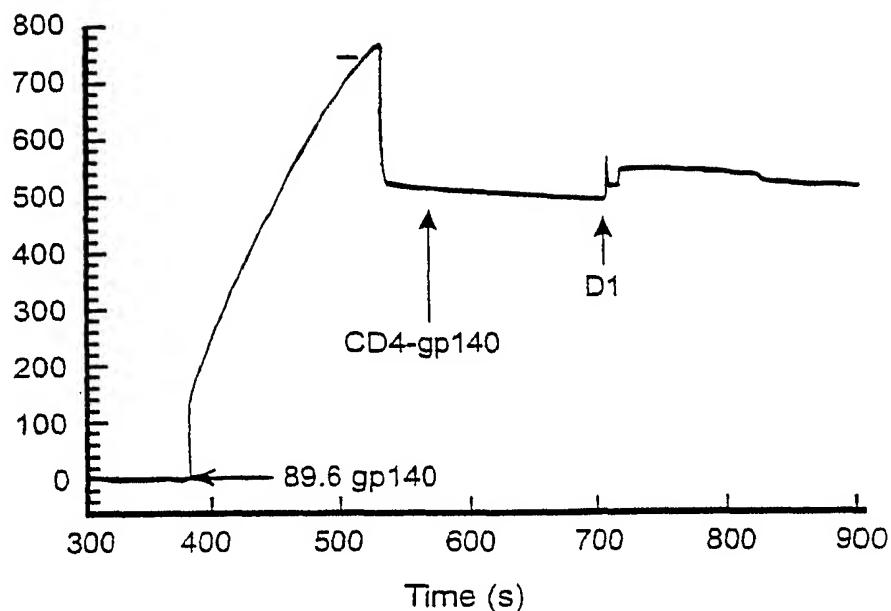


FIGURE 10C

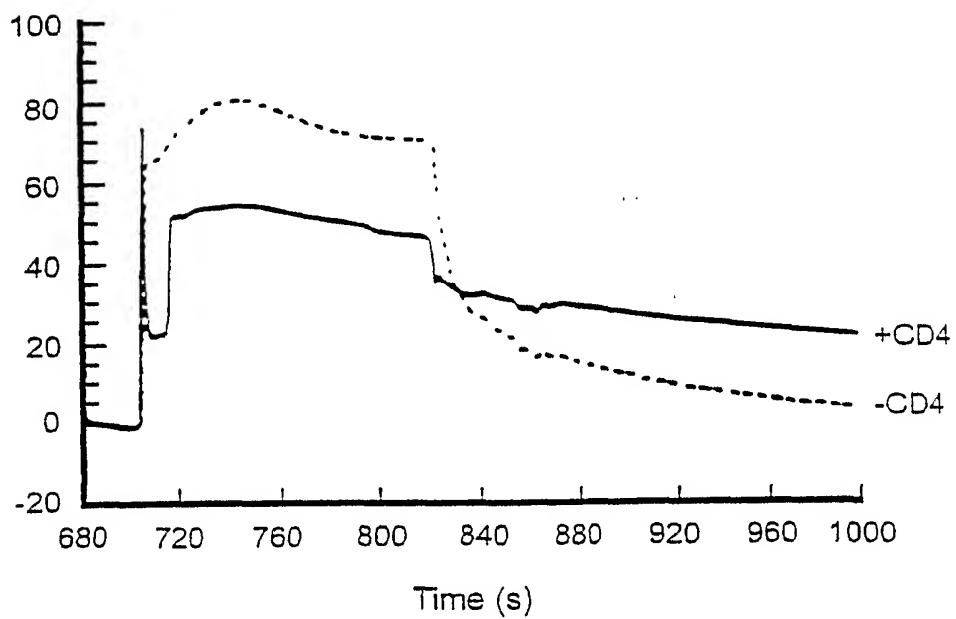


FIGURE 10D

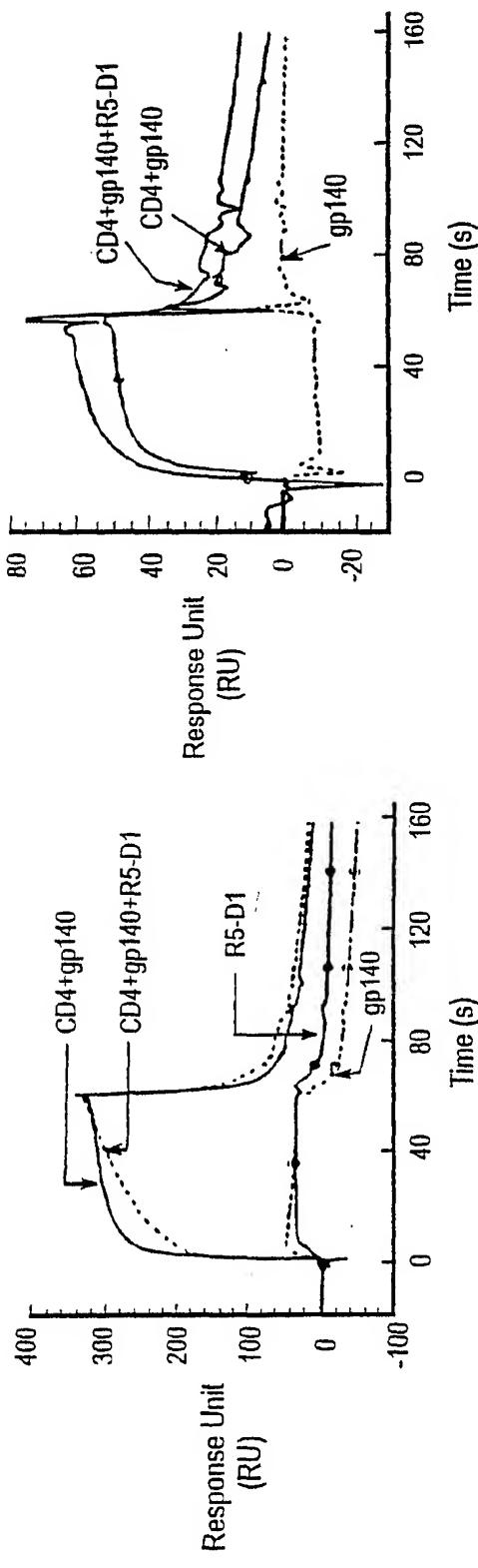


FIGURE 11A

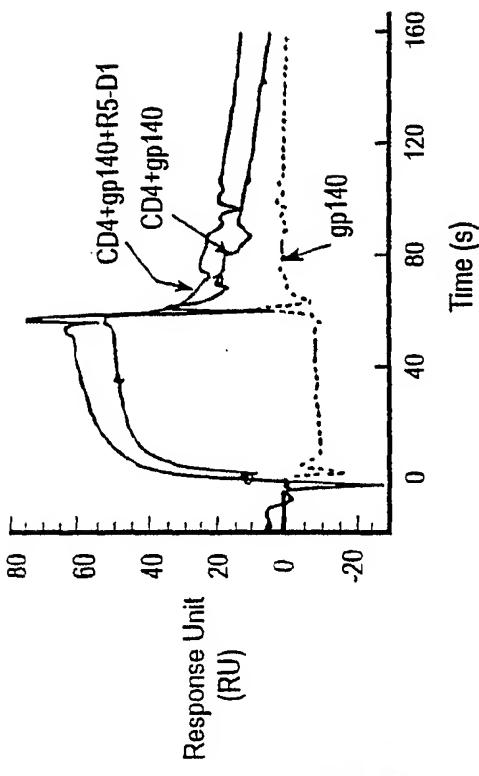


FIGURE 11B

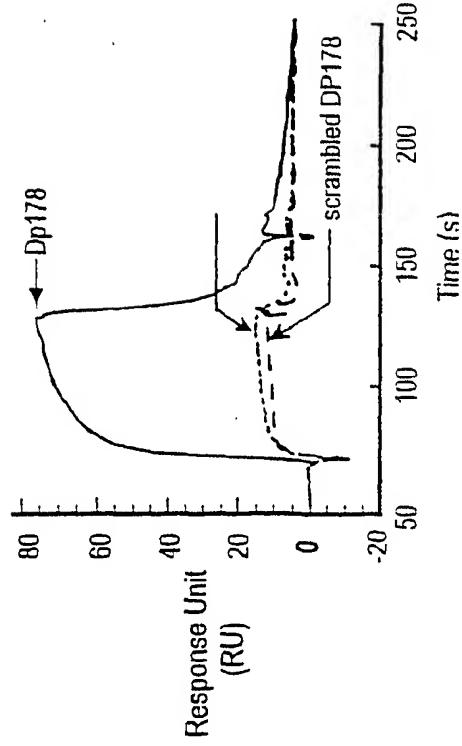


FIGURE 11C

	K _a M ⁻¹ s ⁻¹	K _d s ⁻¹	K _d μM	t _{1/2} s	R _m RU
DP178					
CD4+gp140	7.9X10 ³	5.6X10 ⁻³	0.7	124	320
CD4+gp140+R5-D1	3.47X10 ³	3.86X10 ⁻³	1.1	180	328
gp140+R5-D1	nm	nm	nm	nm	38
gp140	nm	nm	nm	nm	35
T694QL					
CD4+gp140	6.2X10 ³	1.46X10 ⁻²	2.4	48	53
CD4+gp140+R5-D1	5.1X10 ³	5.3X10 ⁻³	1.0	131	64
gp140+R5-D1	nm	nm	nm	nm	0
gp140	nm	nm	nm	nm	0

FIGURE 11D

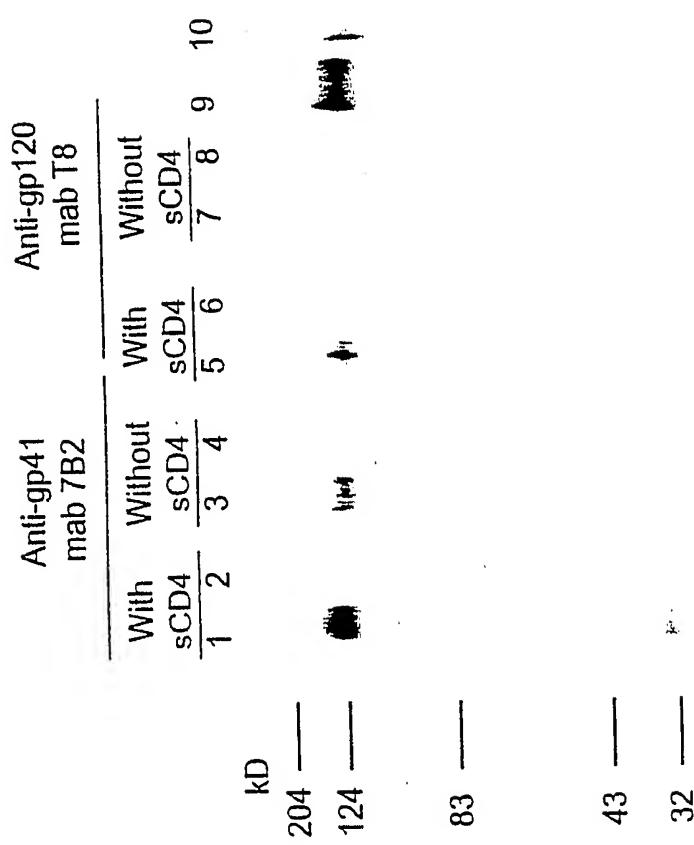
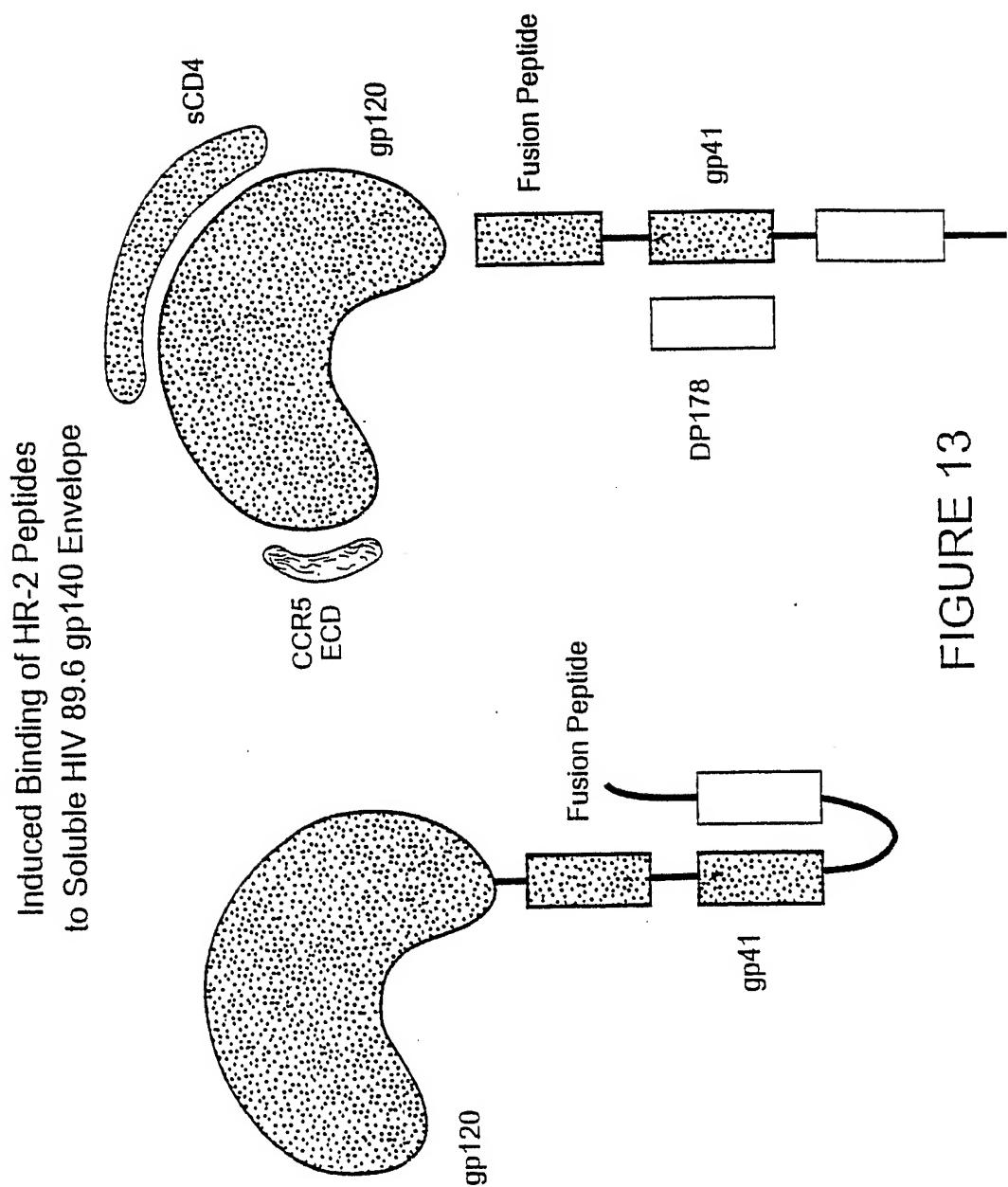


FIGURE 12



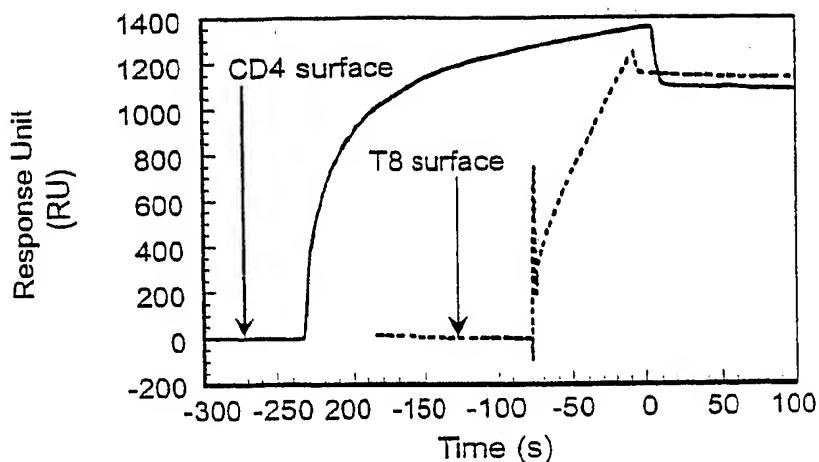


FIGURE 14A

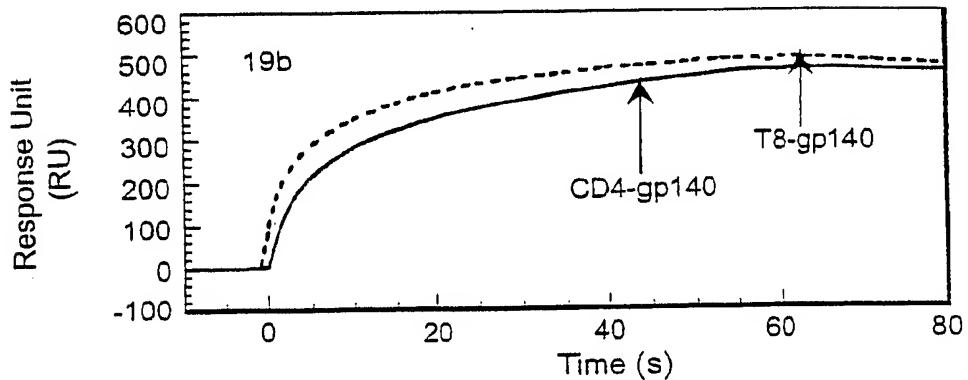


FIGURE 14B

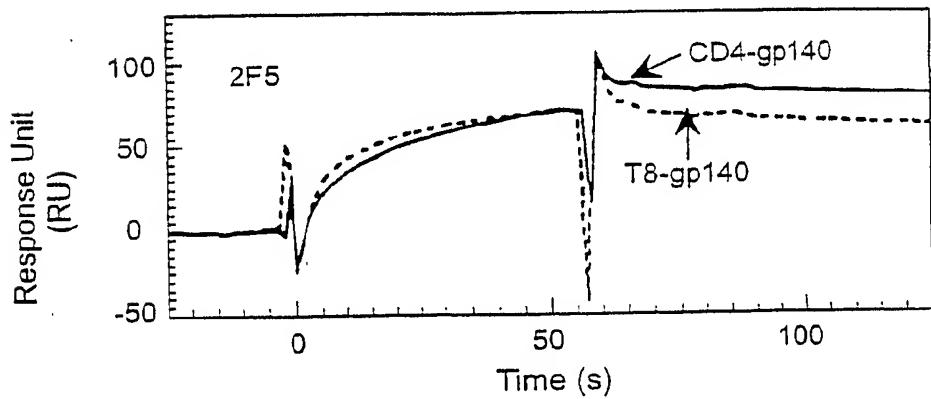


FIGURE 14C

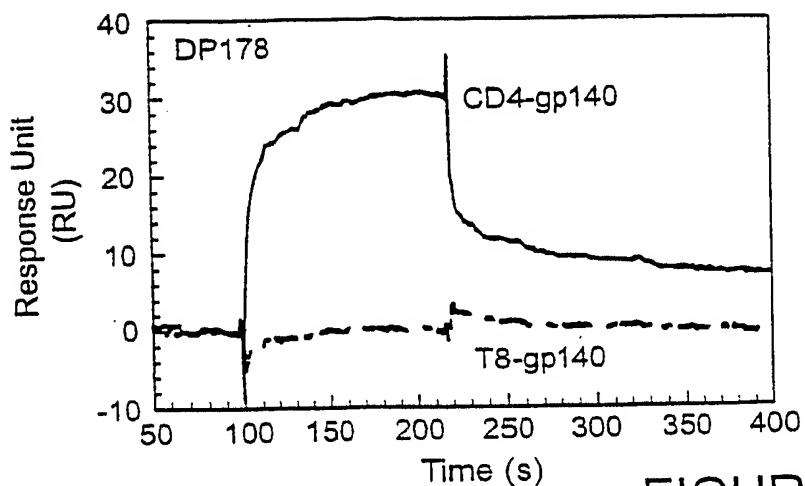


FIGURE 14D

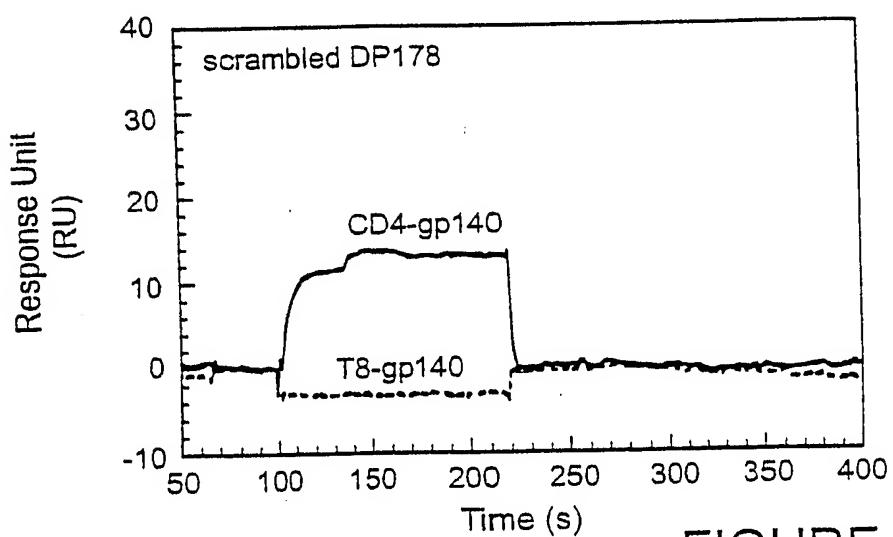


FIGURE 14E

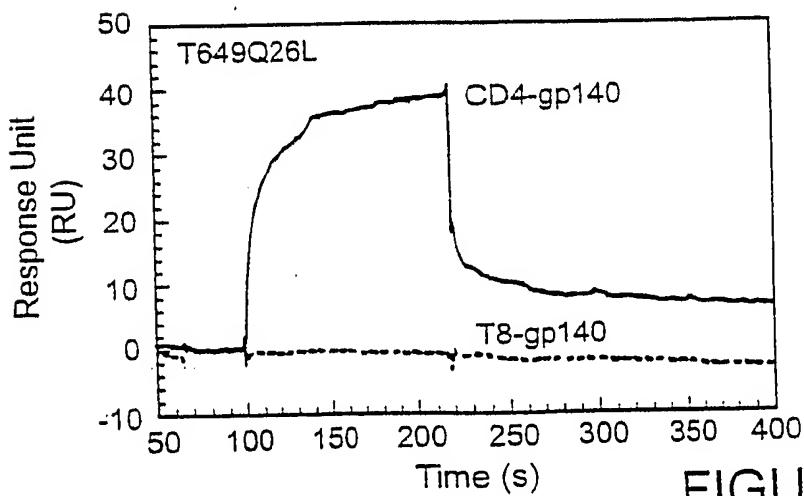


FIGURE 14F

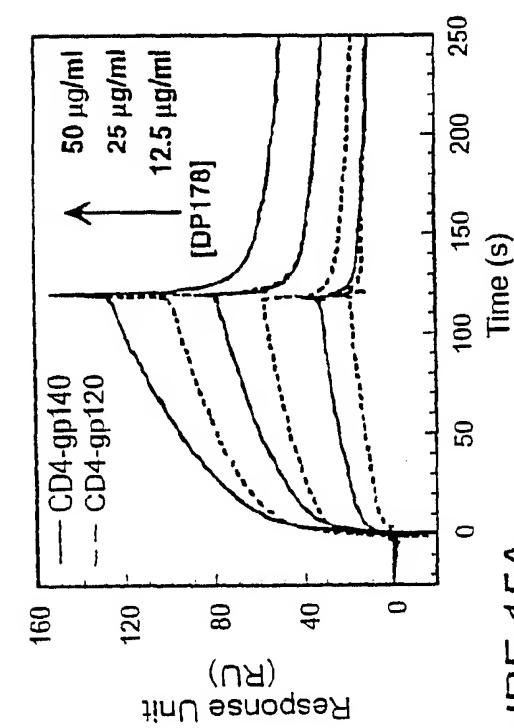


FIGURE 15A

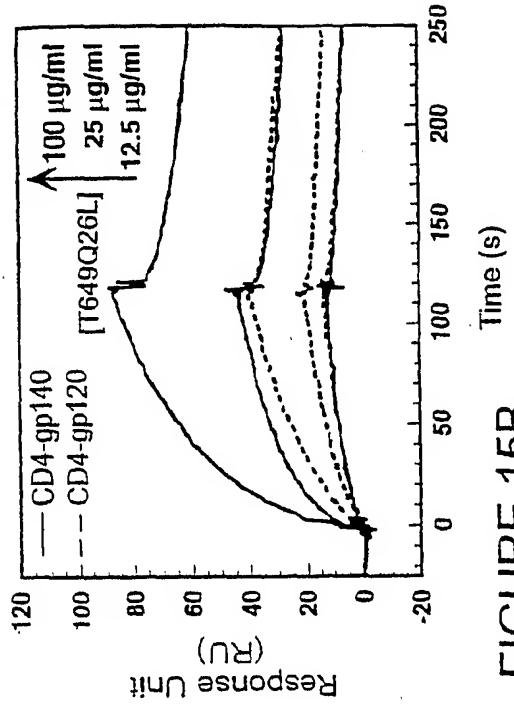


FIGURE 15B

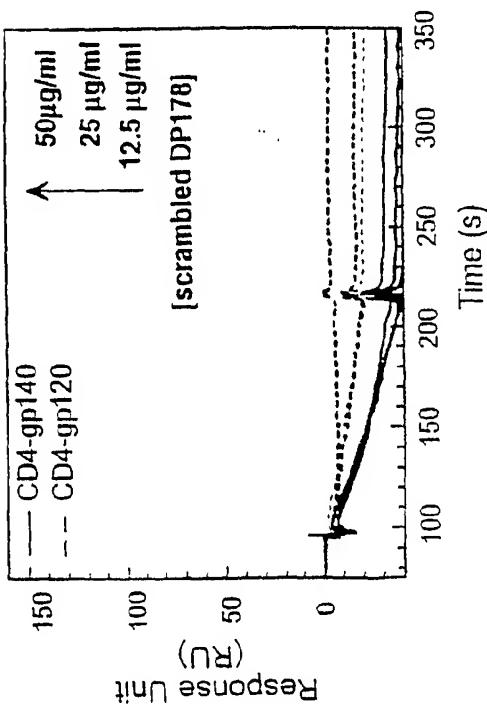


FIGURE 15C

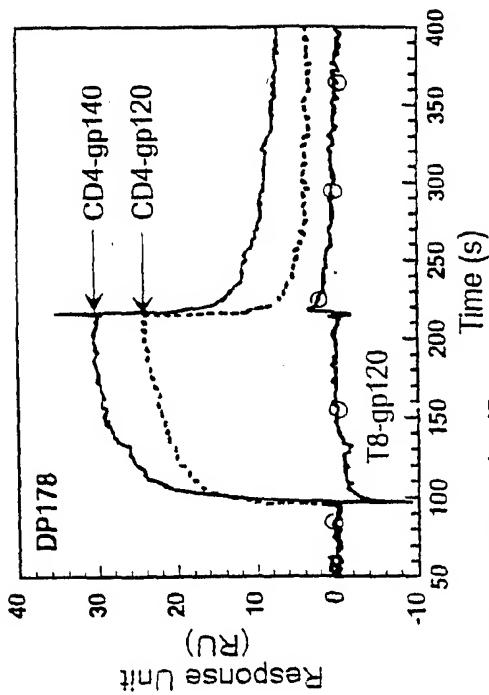


FIGURE 15D

	k_{on} $M^{-1}s^{-1}$	k_{off} s^{-1}	K_d μM	$t_{1/2}$ s
DP178				
CD4-gp120	0.75x10 ³	1.5x10 ⁻³	2.0	462
CD4-gp140	0.93x10 ³	1.0x10 ⁻³	1.1	693
T649Q26L				
CD4-gp120	1.26x10 ³	3.2x10 ⁻³	2.54	217
CD4-gp140	0.88x10 ³	1.1x10 ⁻³	1.20	630

FIGURE 15E

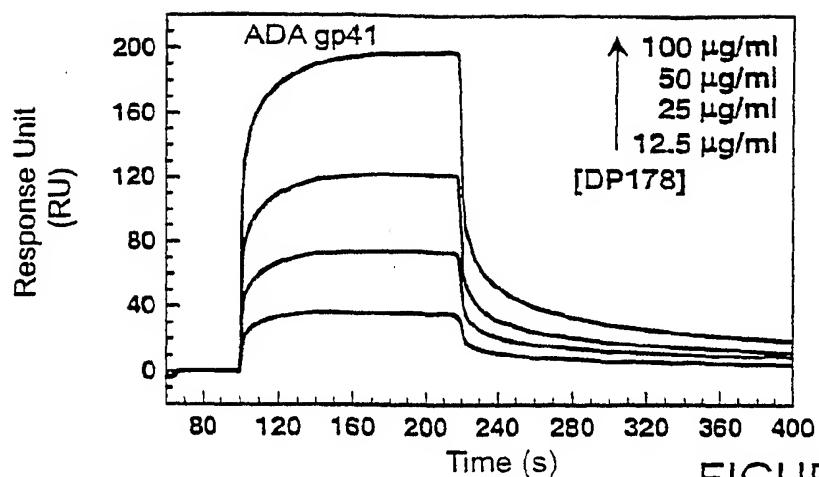


FIGURE 16A

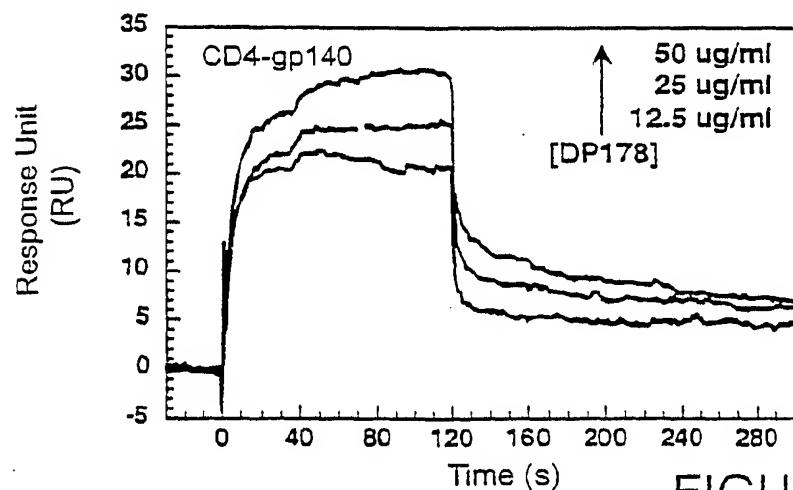


FIGURE 16B

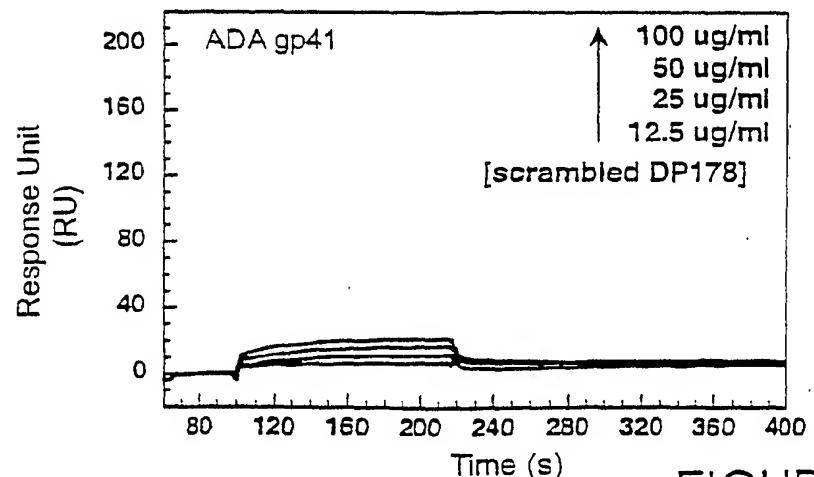


FIGURE 16C

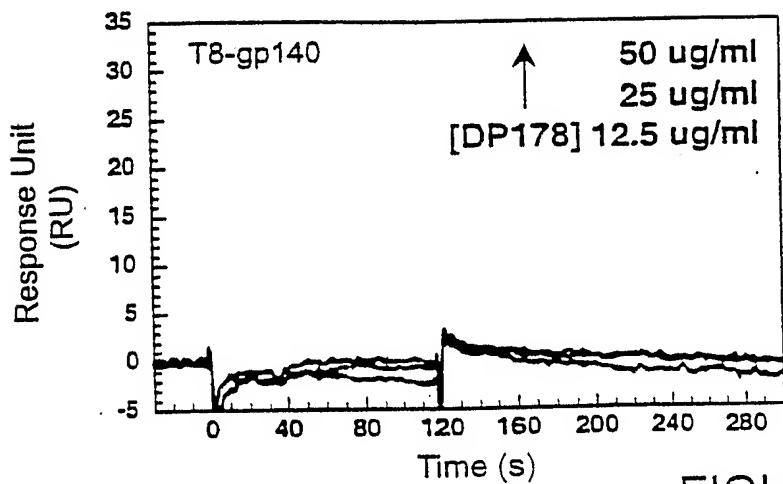


FIGURE 16D

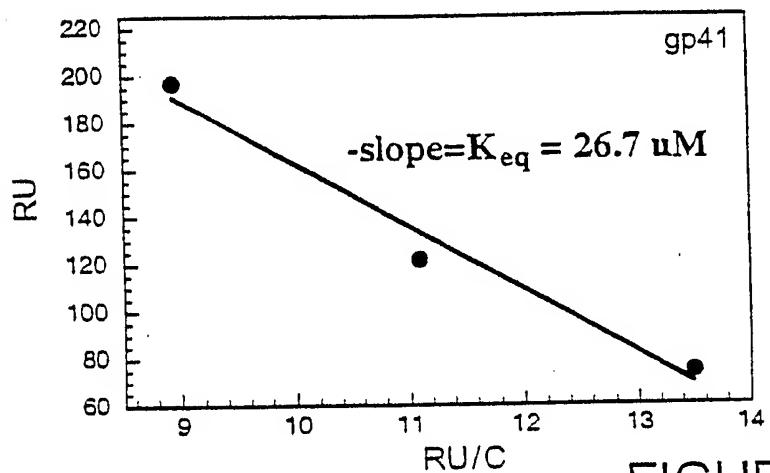


FIGURE 16E

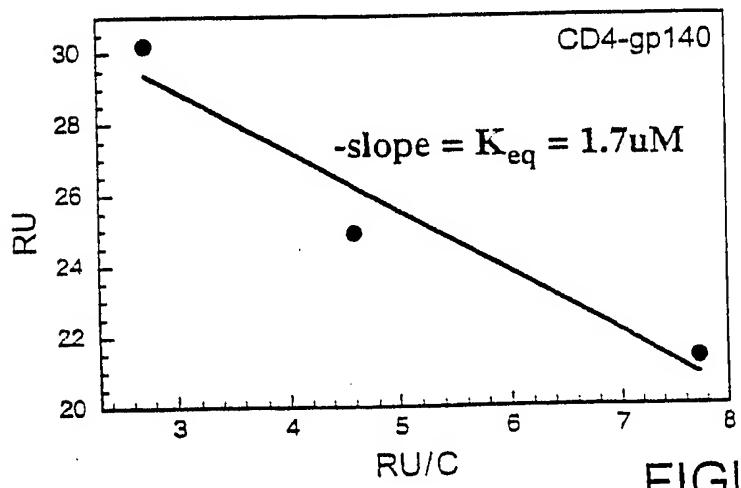


FIGURE 16F

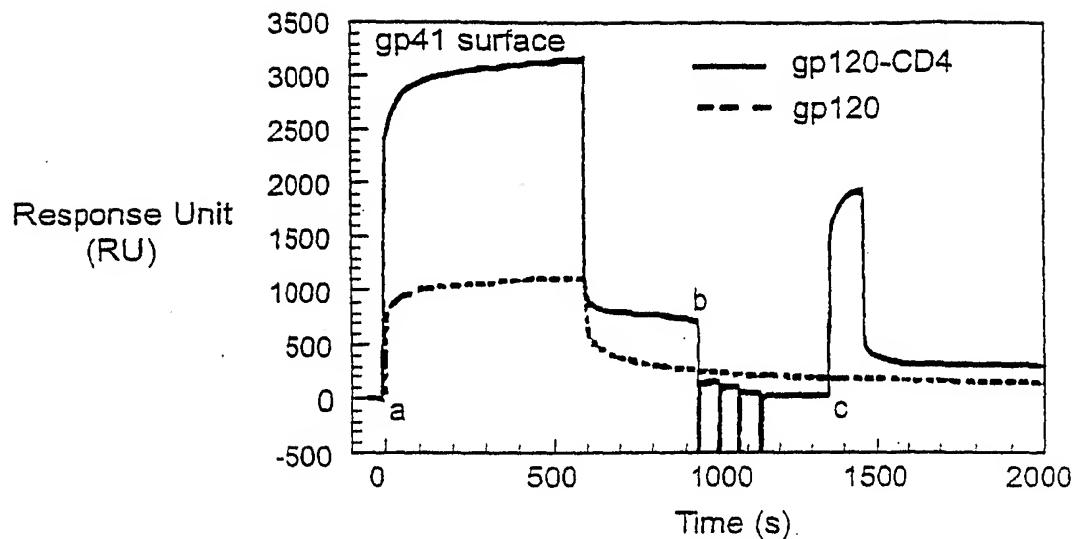


FIGURE 17A

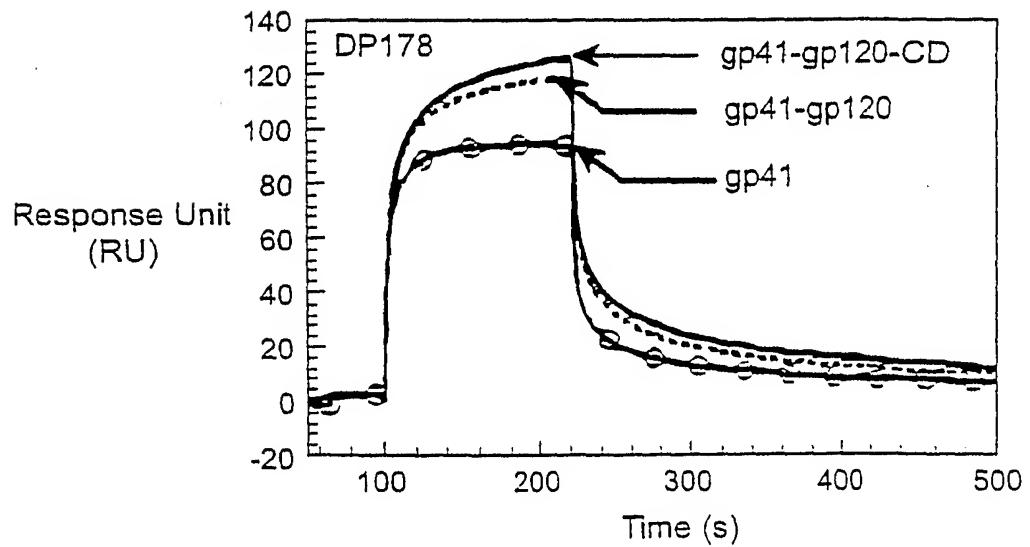


FIGURE 17B

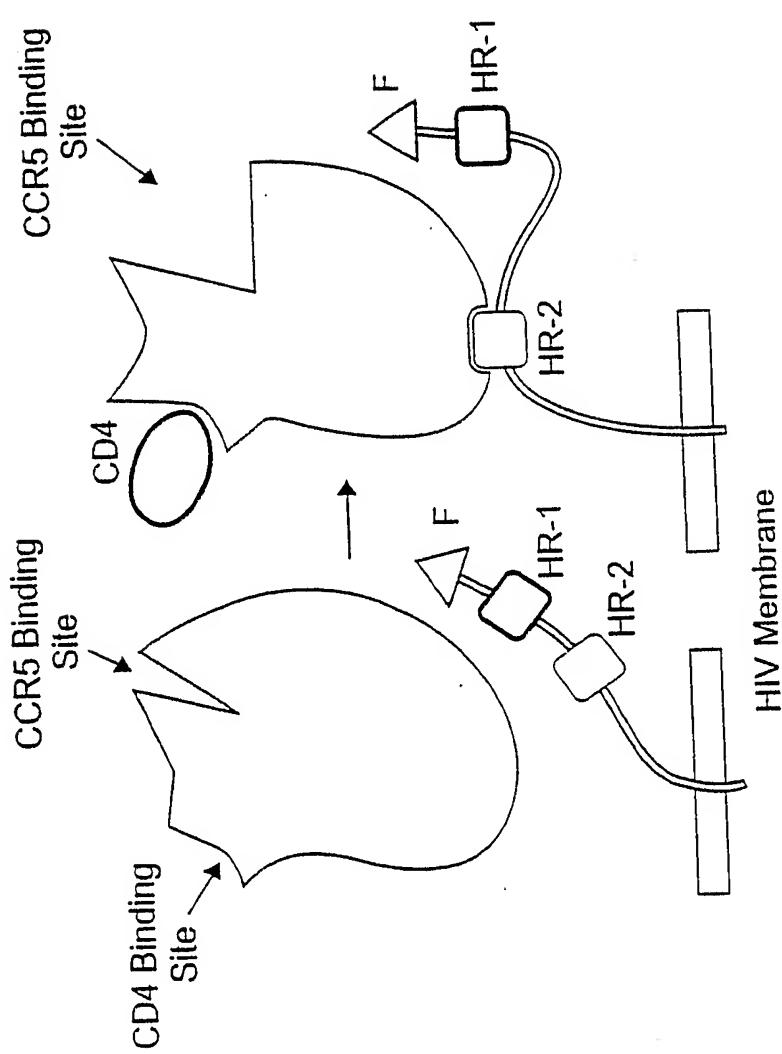


FIGURE 18

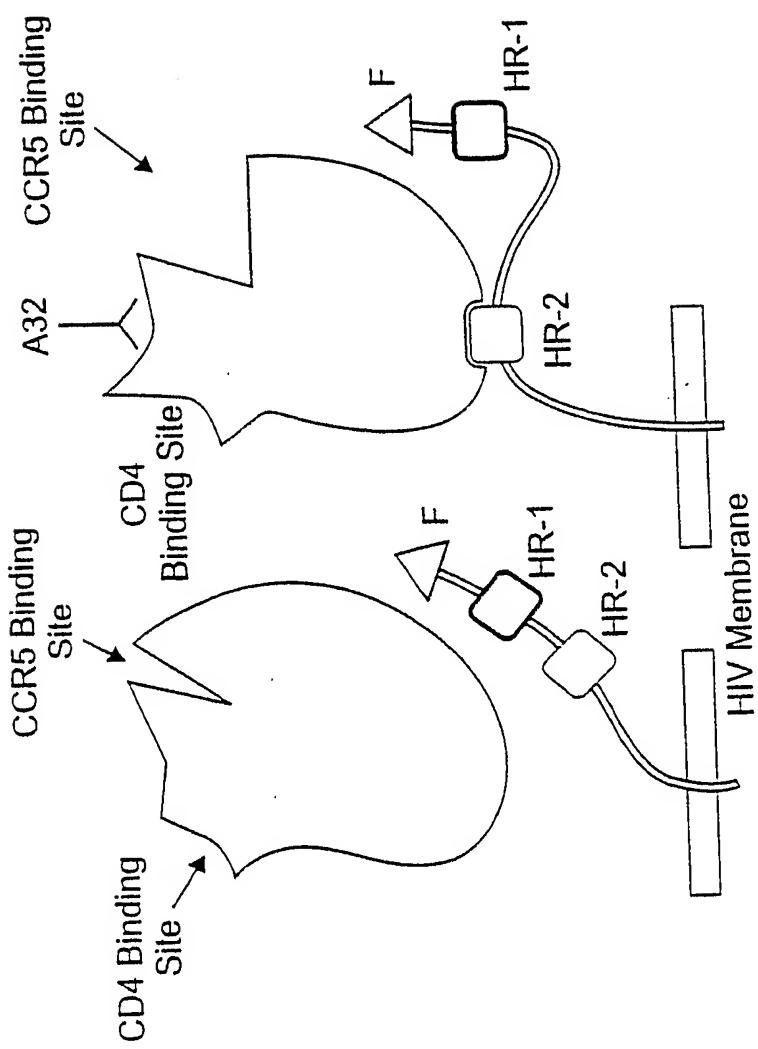


FIGURE 19

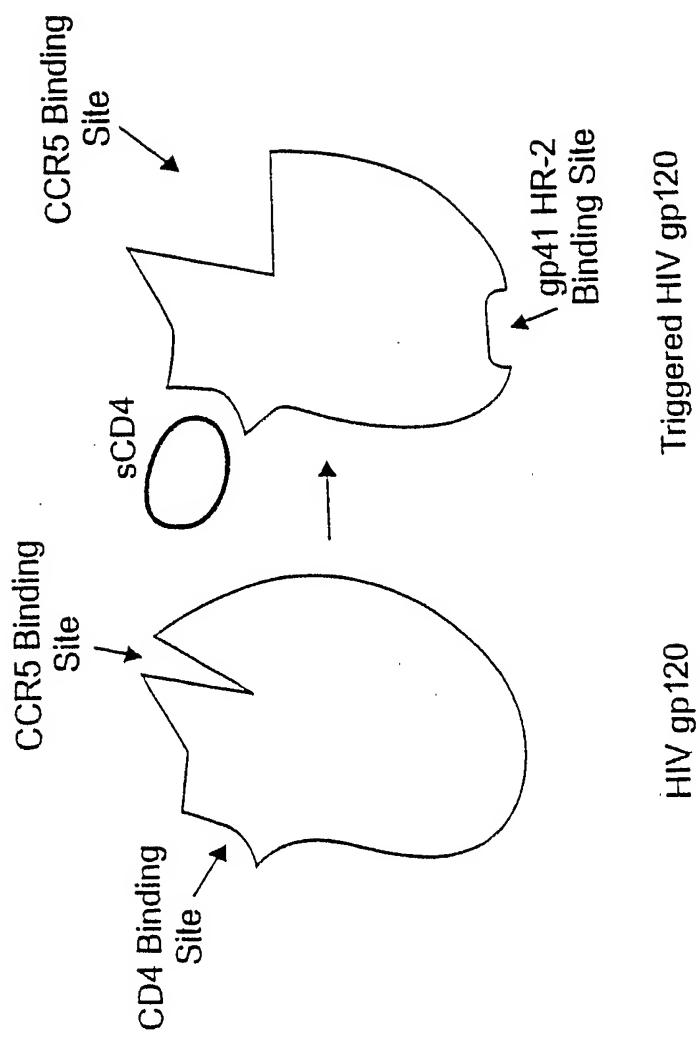


FIGURE 20

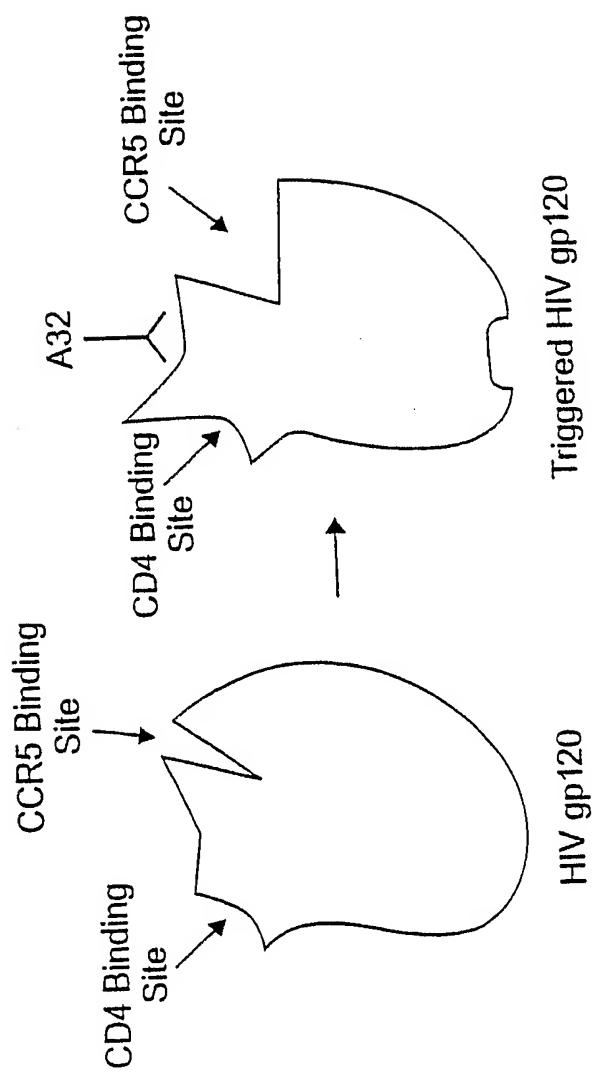


FIGURE 21

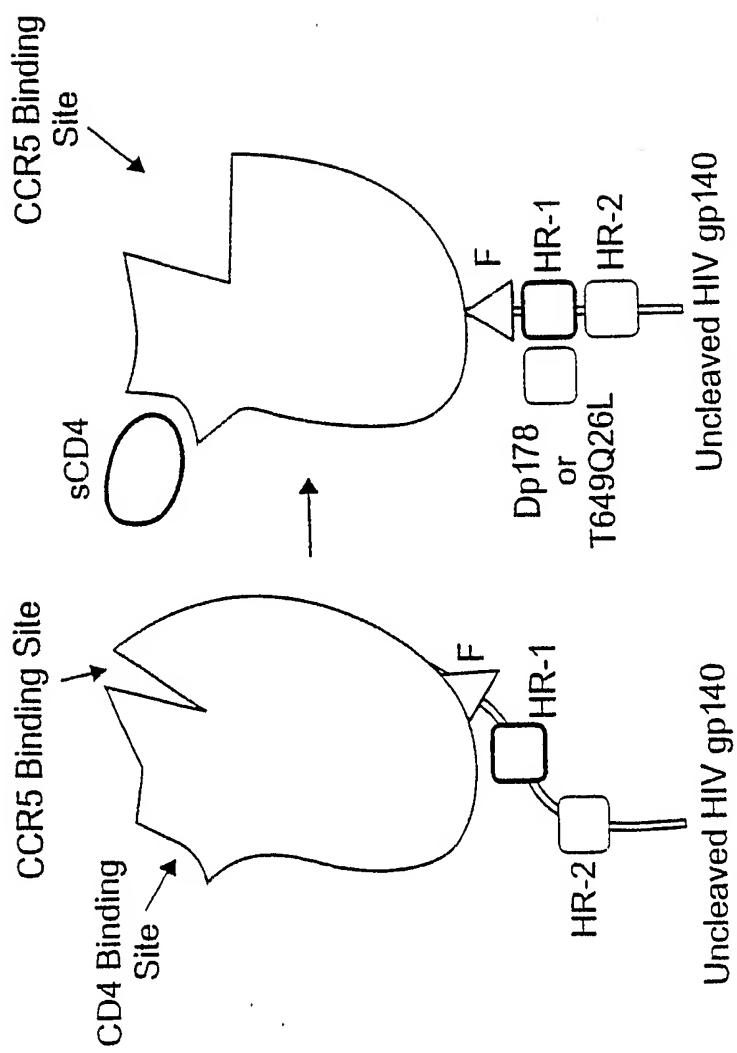


FIGURE 22

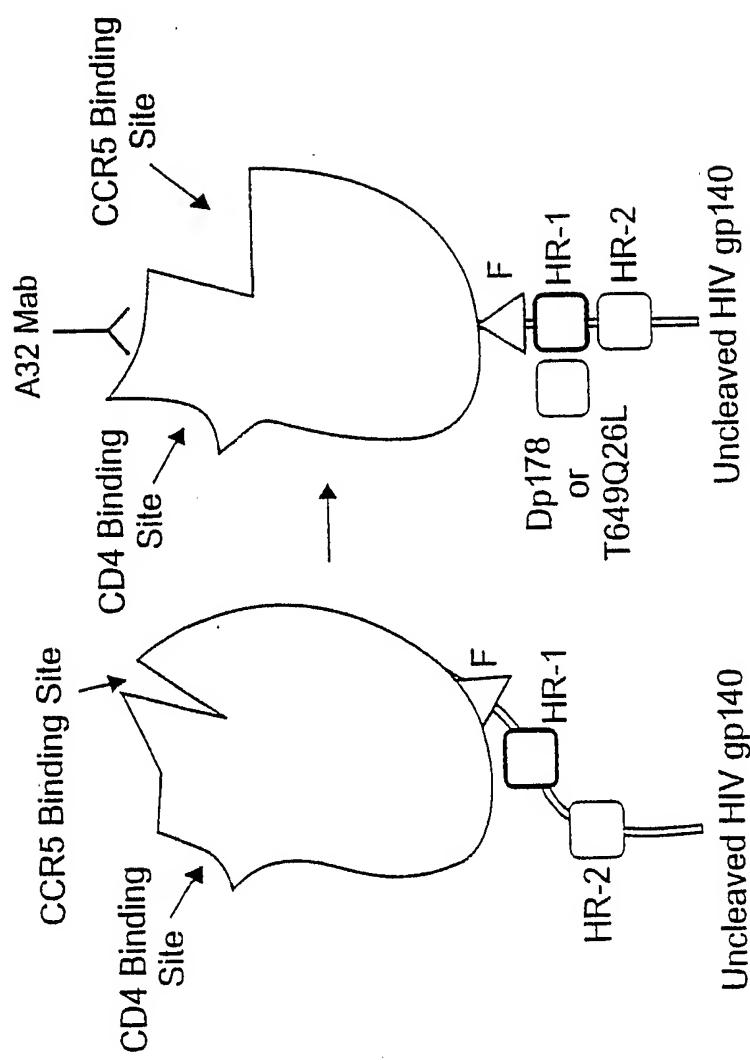


FIGURE 23

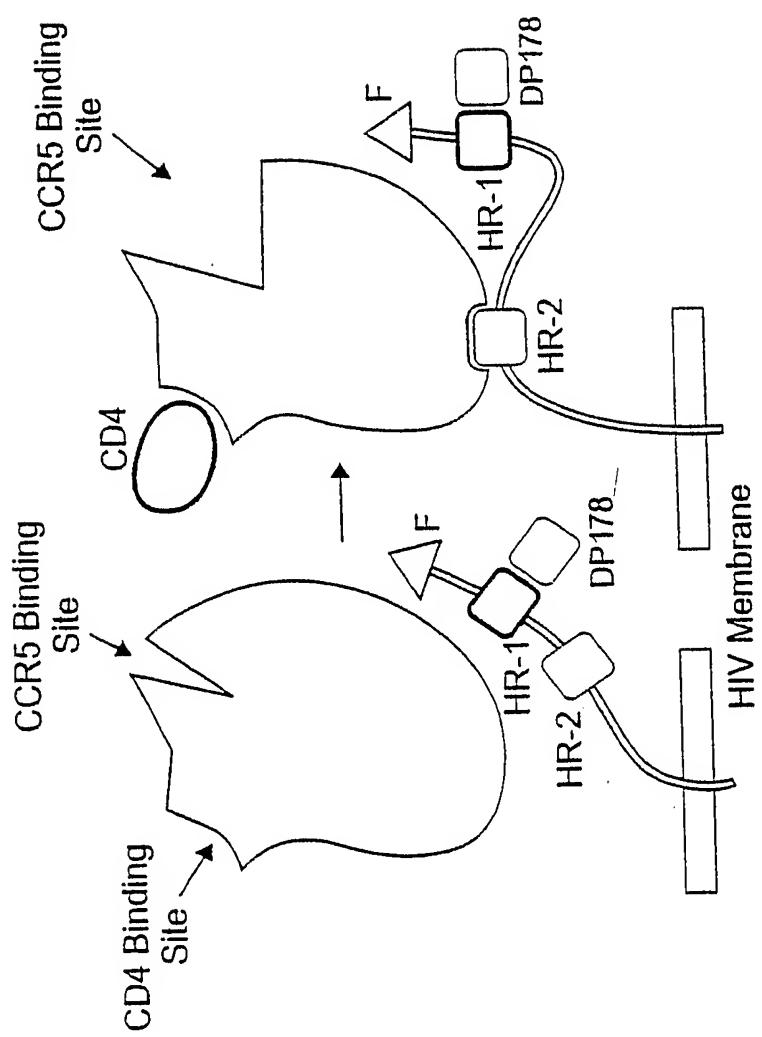


FIGURE 24

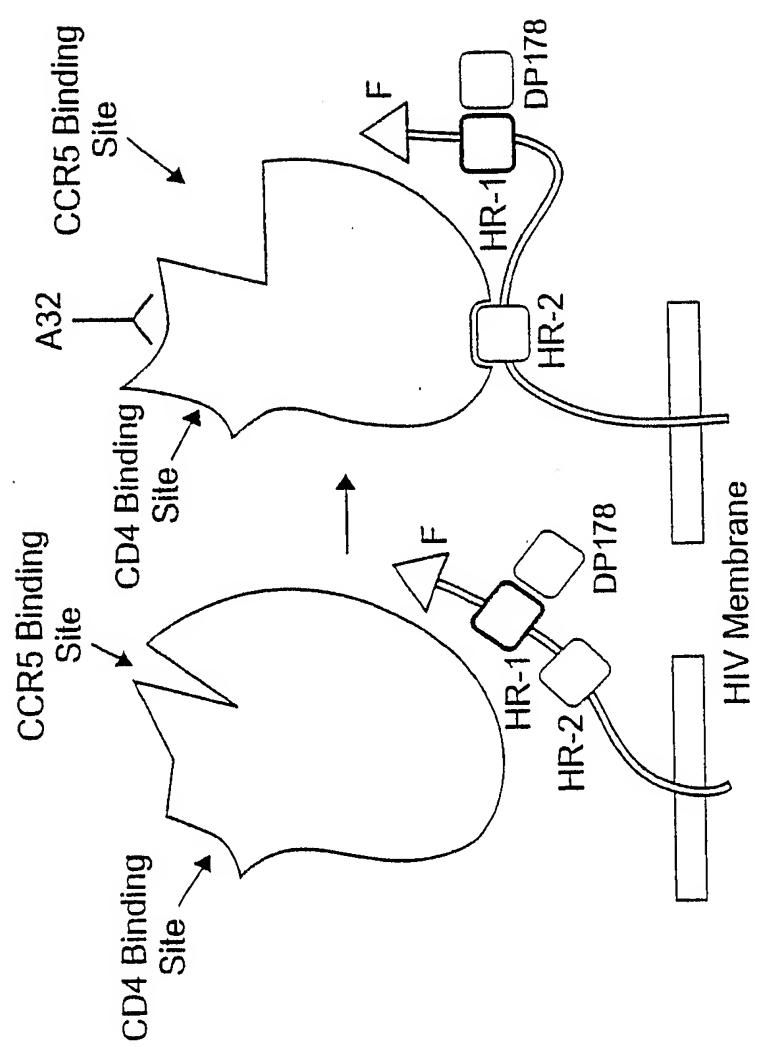


FIGURE 25

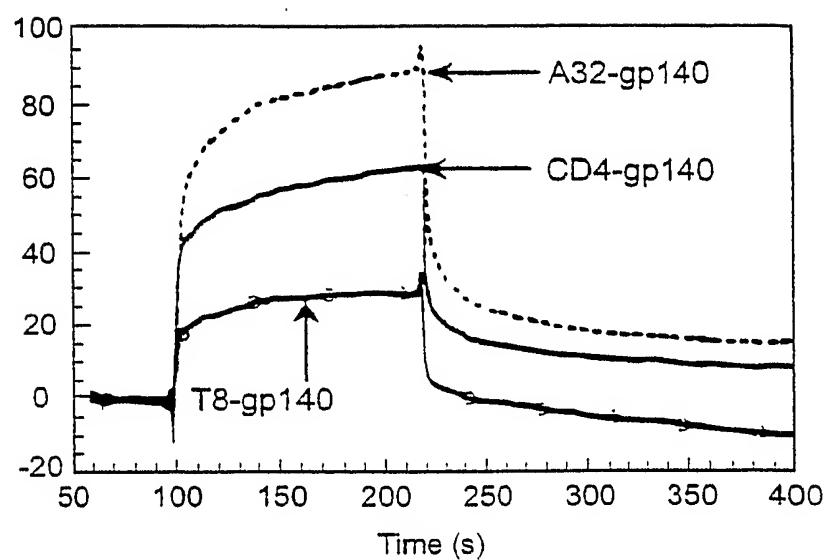


FIGURE 26

Figure 27

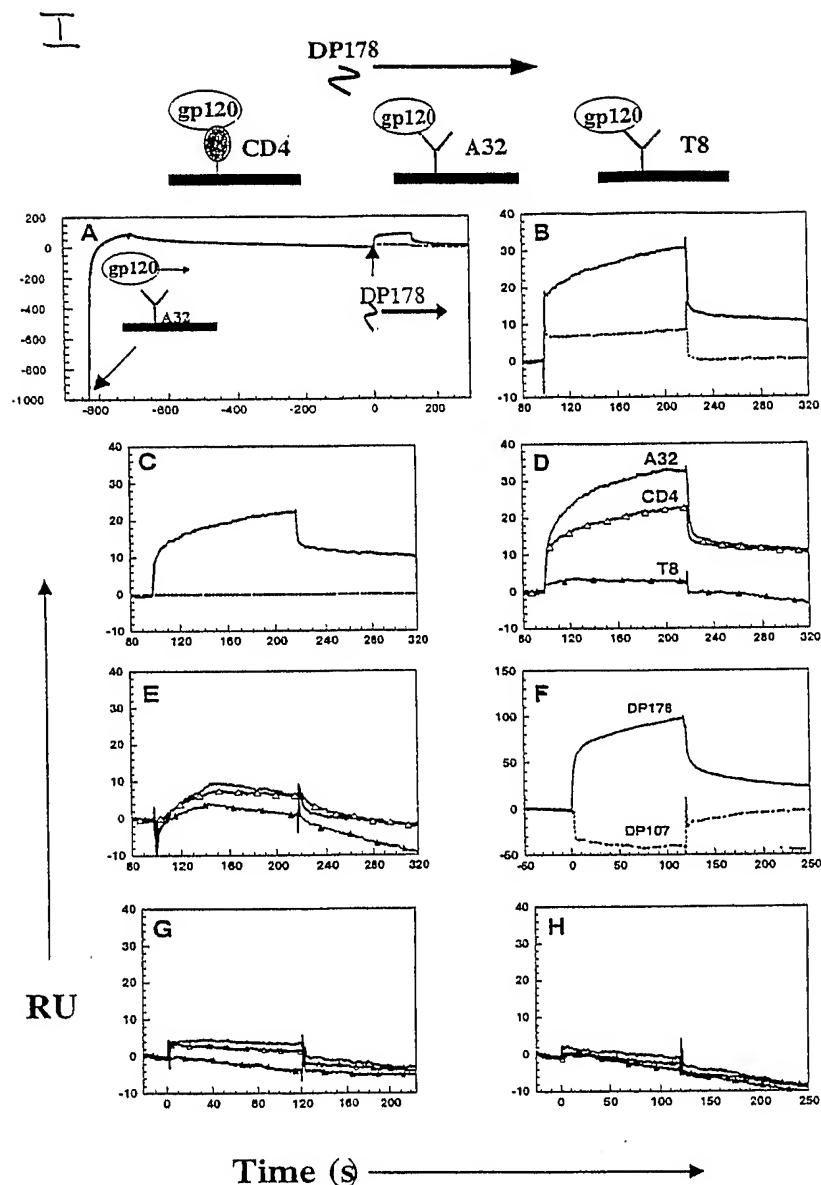
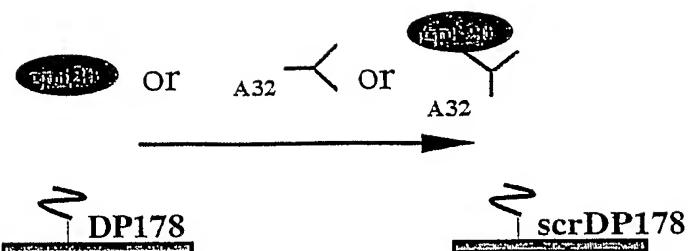
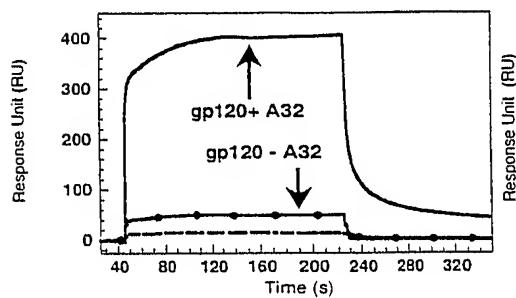


Figure 22

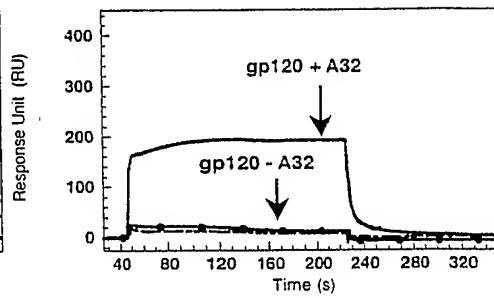
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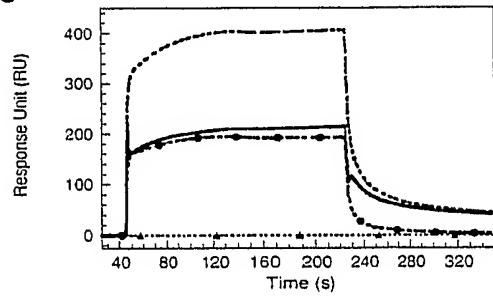
A



B



C



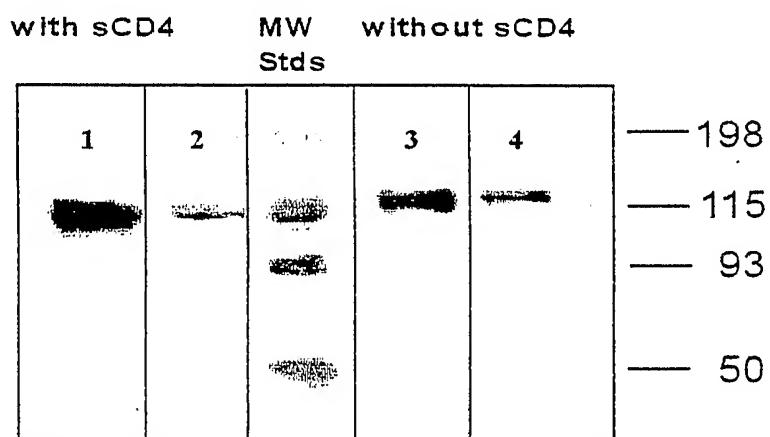
D

	off-rate (s^{-1})	
	k_{d1} (fast)	k_{d2} (slow)
- A32	0.017	-
+ A32	0.020	0.0016

A



B



C

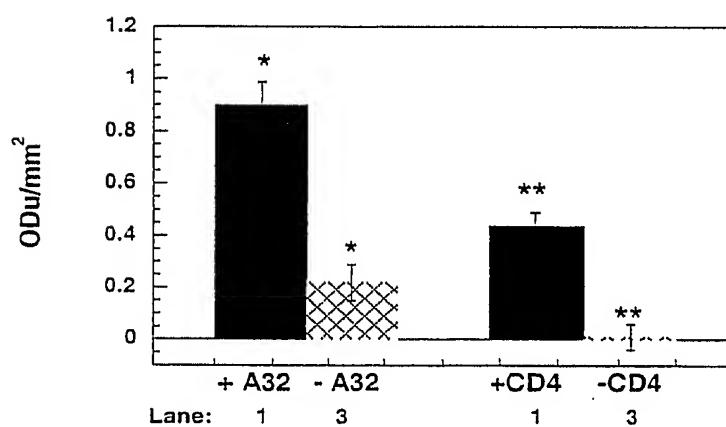


Figure 29

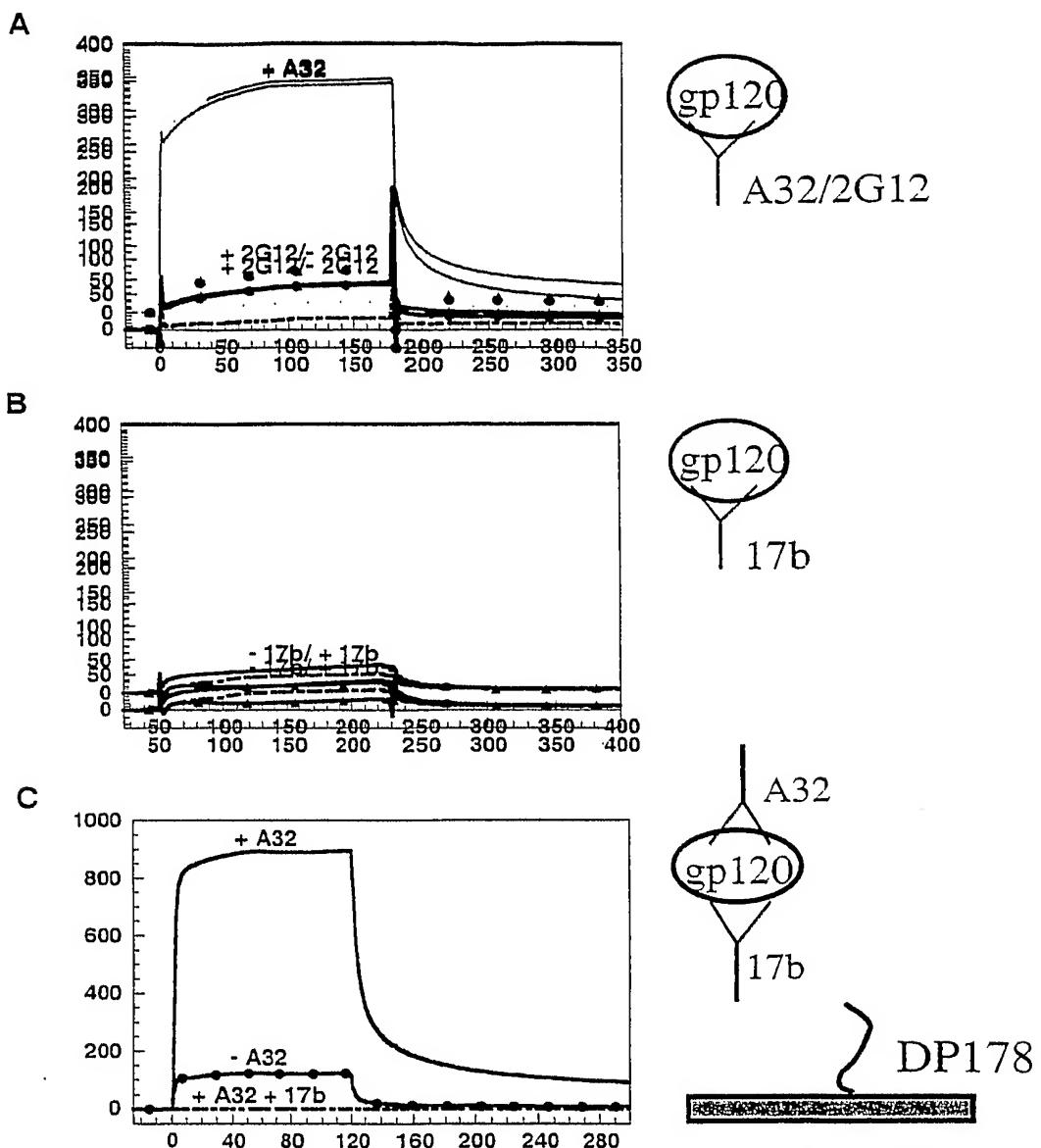


Figure 30

G

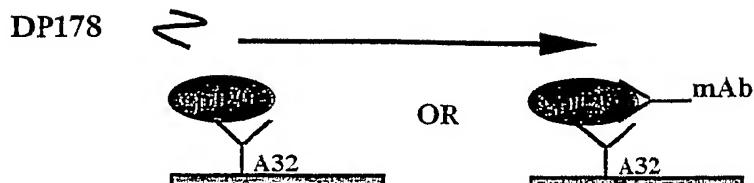
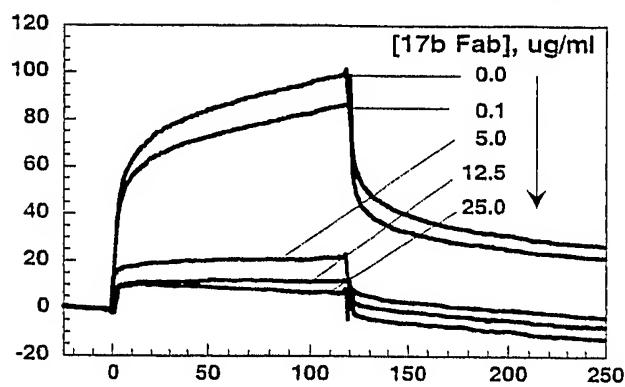
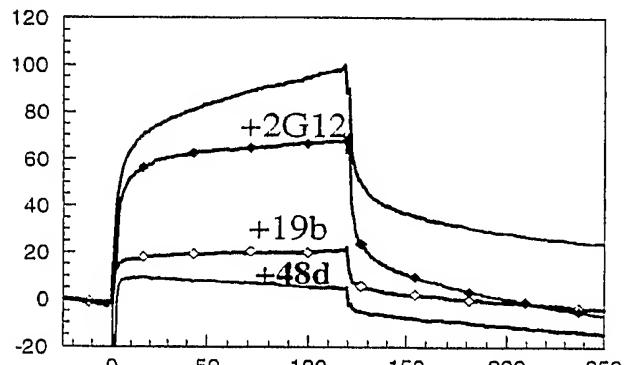


Figure 30

D



M



F

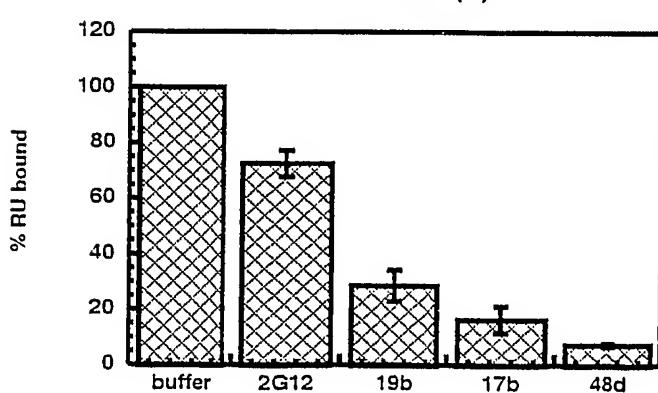
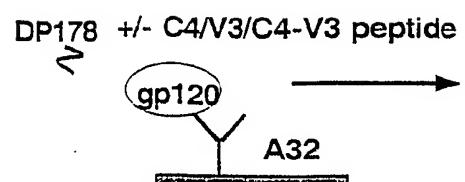


Figure 31

F



RU

